TESIS DOCTORAL

EFECTO DE LA ALDOSTERONA SOBRE LA RESPUESTA VASODILATADORA Y EXPRESIÓN DEL RECEPTOR DEL PÉPTIDO RELACIONADO CON EL GEN DE LA CALCITONINA EN ARTERIAS MESENTÉRICA SUPERIOR Y CEREBRALES DE RATAS NORMOTENSAS E HIPERTENSAS

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OBJETIVOS

MATERIALES Y MÉTODOS, RESULTADOS Y DISCUSIÓN

RESUMEN PUBLICACIÓN 1

PUBLICACIÓN 1: **Márquez-Rodas I**, Longo F, Rothlin RP and Balfagón G. Pathophysiology and therapeutical perspectives of calcitonin gene-related peptide in hypertensión. 2006; Journal of Physiology and Biochemistry 2006;62:45-56

RESUMEN PUBLICACIÓN 2

PUBLICACIÓN 2: Balfagón G, **Márquez-Rodas I**, Álvarez Y, Alonso MJ, Cachofeiro V, Salaices M, Lahera V. Aldosterone modulates neural vasomotor response in hipertensión: role of calcitonin gene-related peptide. Regulatory Peptides. 2004;120:253-60.

RESUMEN PUBLICACIÓN 3

PUBLICACIÓN 3: **Márquez-Rodas I**, Longo F, Aras-López R, Blanco-Rivero R, Diéguez E, Tejerina T, Ferrer M and Balfagón G. Aldosterone increases RAMP1 expression in mesenteric arteries from spontaneously hypertensive rats. Regulatory Peptides 2006;134:61-66

RESUMEN PUBLICACIÓN 4

PUBLICACIÓN 4: **Marquez-Rodas I**, Xavier FE, Villa-Arroyo I, Longo F , Aras-Lopez R, Blanco-Rivero J, Ferrer M and Balfagón G. Increase in CL receptor in cerebral arteries from spontaneously hypertensive rats does not correlate with changes in functional role of CGRP receptor. Regulatory Peptides 2007 (en revisión).

CONCLUSIONES

BIBLIOGRAFÍA

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RESUMEN TESIS

Objetivo: analizamos el efecto de la aldosterona sobre la relajación a CGRP, y sobre la expresión de los componentes de su receptor (CL receptor y RAMP1) en arterias mesentéricas y cerebrales de ratas WKY y SHR. ***Material y métodos:*** medición de tensión isométrica y Western Blot. ***Resultados:*** la aldosterona aumentó la relajación a CGRP y la expresión de RAMP1 sólo en mesentéricas de SHR. En arteria cerebral media, la aldosterona no alteró la vasodilatación a CGRP en ninguna cepa , aunque aumentó la expresión de CL receptor sólo en SHR, sin alterar la de RAMP1. ***Conclusiones***: la aldosterona aumenta la relajación a CGRP en arteria mesentérica de SHR aumentando la expresión de RAMP1. El hecho que en cerebrales no se altere la relajación a CGRP, a pesar de aumentar CL Receptor en SHR, indica que es necesaria la expresión de RAMP1 para que CL Receptor actúe como receptor de CGRP.

Palabras clave: hipertensión; aldosterona; inervación sensitiva; CGRP

Objective: to analyze the effect of aldosterone on CGRP vasodilation and its receptor components expresión (CL Receptor and RAMP1) in mesenteric and cerebral arteries from WKY and SHR rats. ***Methods:*** isometric tension record and Western Blot. ***Results:*** aldosterone increased the vasodilatory effect of CGRP and the expression of RAMP1 in SHR mesenteric arteries only. In middle cerebral arteries, aldosterone did not modify the response to CGRP in either strain, although aldosterone increased the expression of CL Receptor, without modifying RAMP1, in SHR only. ***Conclusions:*** aldosterone increases the vasodilatory effect to CGRP in mesenteric arteries from SHR by increasing the expression of RAMP1. The fact that aldosterone does not alter the effect of CGRP in cerebral arteries, despite of an increase in CL Receptor expression, indicate that RAMP1 is necessary for CL Receptor to function as a CGRP receptor.

Key words: hypertension; aldosterone; sensory innervation; CGRP

INTRODUCCIÓN

El sistema cardiovascular tiene como función principal satisfacer las necesidades de los tejidos (nutricionales, metabólicas, hormonales, inmunes...) para, en definitiva, mantener la homeostasis del organismo. Para ello, el corazón bombea la sangre a través de las arterias y ésta vuelve al corazón por medio de las venas. Aunque la circulación de cada tejido concreto tiene características peculiares, podemos aplicar ciertos principios generales a toda la circulación. Podemos distinguir entre:

- arterias: transportan sangre a los tejidos bajo presiones elevadas
- arteriolas: ramas terminales de las arterias, actúan como válvulas de control del flujo hacia los capilares
- capilares: su función es intercambiar sustancias entre la sangre y el líquido intersticial
- vénulas: recogen la sangre de los capilares, para ir confluyendo en venas cada vez más grandes, hasta retornar la sangre al corazón.

1. ESTRUCTURA DE LA PARED ARTERIAL

Constituida por tres capas (interna, media y externa), la siguiente descripción es aplicable a la arteria mesentérica superior, objeto de nuestro estudio.

1.1 Capa interna o endotelio vascular

Formada por una monocapa de células endoteliales que se apoyan sobre una lámina basal constituida por colágeno IV y AB2, proteoglicanos, glicoproteínas y fibras elásticas, su función va más allá de ser una mera barrera entre sangre y pared vascular (Cabrera Fischer y Armentano, 1995). Así, tiene funciones endocrinas, paracrinas y autocrinas, produciendo y liberando diversas sustancias que contribuyen a la regulación del tono vascular, la respuesta inmune y la coagulación. Entre estas sustancias destacan: óxido nítrico (NO), prostaciclina (PGI2), factor hiperpolarizante derivado de endotelio

(EDHF), endotelina 1 (ET1), prostaglandina H2 (PGH2), tromboxano A2 (TXA2), factores de crecimiento tales como el factor de crecimiento vascular derivado de endotelio (VEDGF), especies reactivas de oxígeno, interleuquinas, activador tisular del plasminógeno (t-PA), etc. (Lahera y col, 1999)

1.2.Capa intermedia o túnica media

Rodea a la interna y está formada fundamentalmente por células de músculo liso. Estas células tienen capacidad contráctil gracias a poseer filamentos de actina y miosina especialmente dispuestos para que, ante diversos estímulos mecánicos o químicos, y mediante un proceso energéticamente activo dependiente de calcio (Ca^{2+}) y tri-fosfato de adenosina (ATP), variar el diámetro interno de la pared vascular, contribuyendo a la regulación de la presión arterial (PA) (Cabrera Fischer y Armentano, 1995; Alberts y col, 1992)

1.3 Capa externa o túnica adventicia

Es la capa más periférica y, al igual que el endotelio, son cada vez más los datos que indican que no es sólo una estructura de soporte inactiva. Formada por colágeno y fibras elásticas, a través de ella pasan los vasa vasorum, que son pequeños vasos que irrigan la pared vascular; y los vasa nervorum, terminaciones nerviosas que liberan distintas sustancias y neurotransmisores que varían según el lecho y la especie analizada (Stefanadis y col, 1995; Cabrera Fischer y Armentano, 1995)

1.3.1 Inervación perivascular

El tono vascular, y consecuentemente la presión arterial, están determinados por un equilibrio entre diversos factores endoteliales, hormonales y nerviosos. Las inervaciones noradrenérgica, serotoninérgica, colinérgica, nitrérgica, peptidérgica y sensitiva, juegan un papel importante en el mantenimiento de este equilibrio en mayor o menor medida, dependiendo del lecho vascular estudiado (Loesch y col, 2002; Marin y col, 2000).

2. ARTERIA MESENTÉRICA SUPERIOR

Esta rama de la aorta abdominal, forma parte de la circulación esplácnica e irriga a intestino delgado y la mitad derecha del intestino grueso (Rouvière y Delmas, 1999, tomo II). Esto supone un gran volumen de sangre circulante que puede desviarse mediante diversos mecanismos, que incluyen la participación de la inervación perivascular, hacia otros tejidos que necesiten una mayor perfusión, constituyendo así un mecanismo importante de la regulación de la PA sistémica (Guyton y Hall, 1996)

La regulación nerviosa de la arteria mesentérica superior es compleja, participando los siguientes tipos de inervación: noradrenérgica, nitrérgica y sensitiva.

2.1 Inervación noradrernérgica

La noradrenalina (NA) se secreta por terminales nerviosas cuyos cuerpos neuronales están en las neuronas postganglionares del sistema nervioso simpático adrenérgico (Matsumura y col 2001). La NA liberada actúa principalmente sobre los siguientes receptores:

- α_1: postsinápticos, en músculo liso vascular, producen vasoconstricción (Arévalo-León y col, 2003)
- α_2: presinápticos, en terminaciones nerviosas perivasculares. Como ya fuera descrito en un principio en gatos (Enero y col, 1972), en la AMS de rata su activación disminuye la liberación de NA, formando así un mecanismo de retroalimentación negativa (homorreceptores) (Yamamoto y Koike, 2001). Por otro lado, la NA puede actuar sobre receptores presinápticos localizados en terminaciones nerviosas distintas de la noradrenérgica (heterorreceptores), modulando la liberación de otros neurotransmisores (Barrús y col, 1990; Marín y Balfagón, 1998)

- β_1: postsinápticos, en las celulas musculares, su activación produce relajación por aumento de la producción de monofosfato de adenosina cíclico (cAMP) (Whalen y col, 1998)
- β_2: presinápticos, en terminaciones noradrenérgicas de ratas hipertensas, su activación aumenta la liberación de NA (Misu y col, 1987). En las terminaciones nitrérgicas, su activación produce un aumento de la liberación de NO (Marín y Balfagón, 1998)

En general, la NA tiene más afinidad por los receptores α_1 que por los β_1, lo que hace que, debido al predominio de receptores $\alpha 1$ en el músculo liso, el efecto neto de la estimulación de las fibras noradrenérgicas sea vasoconstrictor (Vanhoutte y col, 1981).

En numerosos lechos vasculares, se ha demostrado que la NA se coalmacena y colibera con otros neurotransmisores tales como el neuropéptido Y (NPY) y el ATP. El NPY actúa sobre receptores Y1 postsinápticos, produciendo vasoconstricción, y sobre Y2 presinápticos, produciendo disminución de la liberación de NA (Donoso y col, 2004). Y el ATP actúa sobre receptores PX2 pstsinápticos produciendo vasoconstricción (Burnstock, 2006).

2.2 Inervación nitrérgica

Libera NO como neurotransmisor, que es un gas lábil sintetizado por la óxido nítrico sintasa (NOS), a partir de L-arginina, existiendo tres isoformas de esta enzima (Alderton y col, 2001):

- Formas dependientes de calcio/calmodulina: Son constitutivas
    - neuronal, nNOS o NOS I
    - endotelial, eNOS o NOS III
- Forma independiente de calcio/calmodulina: Es inducible, iNOS o NOS II.

En arteria mesentérica de rata, la activación de receptores β_2-adrenérgicos presentes en la inervación nitrérgica produce la liberación de NO (Marín y Balfagón,1998) produciendo relajación de las células musculares lisas por aumento de los niveles de monofosfato de guanosina cíclico (cGMP) (Iramani, 1997)

2.3 Inervación sensitiva

El núcleo de estas fibras se localiza en los ganglios dorsales de la médula espinal, localizándose las terminaciones entre la adventicia y la media de los vasos, principalmente arterias (Gulbekian y col,1993). Liberan diversos neuropéptidos, siendo el principal el péptido relacionado con el gen de la calcitonina (CGRP), liberando también en forma de cotransmisores la sustancia P (SP) y neurokinina A (NKA) principalmente (Gangula y col, 2000). El estudio de la función del CGRP es uno de los objetivos centrales de este trabajo, y se exponen de forma detallada su estructura, receptores, propiedades y papel en la regulación del tono vascular y PA más adelante.

3. CIRCULACIÓN CEREBRAL

Las arterias del encéfalo se originan a partir de las arterias vertebrales y las carótidas internas. Las vertebrales se unen para formar la arteria basilar, la cual se divide en dos arterias cerebrales posteriores. Las carótidas internas, lateralmente al quiasma óptico, originan las arterias cerebrales medias, anteriores y comunicantes posteriores. Estas últimas se unen a las arterias cerebrales posteriores para, junto a la arteria comunicante anterior, formar una estructura circular denominada polígono de Willis. Esta disposición asegura la circulación cerebral en caso de obliteración de uno de los troncos carotídeos o vertebrales (Rouvière y Delmas, 1999, tomo III).

Cada una de las ramas irriga una parte del cerebro, de forma que la oclusión de unas u otras derivará en un déficit neurológico focal con consecuencias distintas según el lugar que irriguen.

La circulación cerebral tiene una característica especial, que es su capacidad de autorregulación del flujo. Así, en condiciones de salud, el flujo sanguíneo cerebral puede mantenerse constante entre unos valores de presión amplios, de tal manera que cambios en la PA no alteran el flujo cerebral de forma significativa. Sólo cuando se alcanzan valores muy altos o muy bajos de PA pueden provocar un aumento o descensos bruscos de la perfusión cerebral (Guyton y Hall, 1996).

En el mantenimiento del flujo cerebral participan factores musculares de la pared vascular, metabólicos, hormonales y nerviosos (Edvinsson y Krause, 2002). De la misma forma que la arteria mesentérica superior de rata, las arterias cerebrales están inervadas por diversos tipos de terminaciones nerviosas que, por medio de sus distintos neurotransmisores, participan en la regulación del tono vascular. En el control de la circulación cerebral de rata participan:

3.1 Inervación noradrenérgica

La mayoría de las fibras se originan en el ganglio cervical superior (Iwayama y col, 1970) siendo la inervación más densa en los vasos anteriores y en los de mayor calibre (Edvinsson y Krause, 2002). La NA, actuando sobre receptores α_1 adrenérgicos postsinápticos, produce una potente vasoconstricción, lo que disminuye el flujo sanguíneo cerebral (Edvinsson y Uddman, 2005). Como en la arteria mesentérica superior de rata, se ha descrito la existencia de receptores α_2 adrenérgicos presinápticos, que producen una disminución de la liberación de NA (Edvinsson y Krause, 2002)

3.2 Inervación colinérgica

Su neurotransmisor es la acetil-colina (Ach), la cual produce vasodilatación por la activación de receptores M1 muscarínicos endoteliales, produciendo un aumento de la producción de NO. Sus fibras provienen de los ganglios esfenopalatino y ótico (Edvinsson y Krause, 2002).

3.3 Neuropéptido Y

Ampliamente distribuido en la inervación perivascular, se encuentra principalmente como cotransmisor de la NA, produciendo vasoconstricción al activar receptores específicos. Parece que su papel fisiológico es el de optimizar la función de la NA (Edvinsson y Krause, 2002)

3.4 Inervación nitrérgica

Sus fibras provienen de núcleos intracraneales tales como el ganglio ótico, esfenopalatino y ganglio de Gasser o trigeminal, y rodean a los vasos cerebrales. (Edvinsson y Krause, 2002). Sus efectos son los mismos que los descritos para la arteria mesentérica superior.

3.5 Inervación serotoninérgica

La 5 hidroxi-triptamina (5HT, serotonina) es un potente vasoconstrictor presente en varios tipos de células, tales como neuronas y plaquetas. El origen de las fibras serotoninérgicas perivasculares cerebrales no está claro. Mientras unos defienden que se originan en los núcleos del rafe (Bovento y col, 1989), otros consideran que proviene de la recaptación por parte de las fibras noradrenérgicas de la 5HT circulante, que luego sería liberada como falso neurotransmisor (Chang y col, 1989)

3.6 Inervación sensitiva

Las fibras sensitivas que inervan las arterias cerebrales provienen del ganglio de Gasser o trigeminal y contienen, al igual que en la arteria mesentérica superior, SP, NK y sobre todo CGRP (Edvinsson y Krause, 2002). Como referimos en el apartado de la arteria mesentérica superior, se exponen de forma detallada su estructura, receptores, propiedades y papel en la regulación del tono vascular y PA más adelante, en la publicación 1.

4. REGULACIÓN DE LA PRESIÓN ARTERIAL

El mantenimiento PA dentro de unos límites normales garantiza la adecuada perfusión de los distintos organos y tejidos. La PA está relacionada con el gasto cardiaco (GC) y la resistencia periférica (RP), de forma directamente proporcional (PA = GC x RP). El organismo ha desarrollado varios mecanismos que permiten controlar la PA, que podemos dividir en dos grandes grupos: mecanismos de control rápido, en los que participan los efectos del sistema nervioso autónomo y de sustancias producidas por el metabolismo general y local; y mecanismos de control a largo plazo, formados por el sistema renal y el sistema renina-angiotensina-aldosterona.

4.1 Regulación nerviosa de la PA: control rápido

Intervienen sobre todo el sistema simpático y la médula suprarrenal, y en menor medida el sistema parasimpático.

- Sistema simpático: como hemos indicado anteriormente, la mayoría de vasos sanguíneos están inervados por fibras simpáticas adrenérgicas. Mediante la liberación de NA producen vasoconstricción en arterias y arteriolas, produciendo redistribución del flujo; y en venas la contracción aumenta el retorno venoso al corazón. Además, la inervación simpática produce sobre el corazón un aumento de la frecuencia y fuerza de contracción, aumentando el GC.
- Médula adrenal: esta parte de las glándulas suprarrenales se encarga de producir y liberar catecolaminas (adrenalina, noradrenalina). La liberación aumenta gracias a la estimulación del sistema simpático, contribuyendo así al aumento rápido de la PA
- Sistema parasimpático: de menor importancia en la regulación del tono vascular periférico, sobre todo es importante por su acción sobre el corazón. Así, la acetilcolina (Ach, principal neurotransmisor) produce los efectos contrarios a las

catecolaminas en el corazón. De esta manera, ambos sistemas participan en el mantenimiento de la PA (Guyton y Hall, 1996)

4.2 Regulación a largo plazo de la PA: el sistema renina-angiotensina-aldosterona

En el control a largo plazo de la PA se realiza gracias al mantenimiento de la cantidad de agua (H_2O) y sodio (Na^+) extracelulares y del tono vascular. En este proceso tienen un papel predominante los riñones y el sistema renina-angiotensina-aldosterona.

Brevemente, los riñones regulan la cantidad de H_2O y Na^+ extracelular mediante el control de la diuresis, de forma que cuando aumentan, aumenta la PA, aumentan la diuresis y la natriuresis, y viceversa. Además de este control, los riñones tienen otro mecanismo importante del control de la PA: el sistema renina-angiotensina-aldosterona.

Cuando la PA disminuye en exceso, las células del aparato yuxtaglomerular de la nefrona detectan esta caída, liberando renina al torrente sanguíneo. Esta enzima actúa sobre el angiotensinógeno circulante en el plasma, que se convierte en angitensina I, de ligero efecto vasoconstrictor. La angiotensina I es convertida gracias a la enzima convertidora de angiotensina (ECA), localizada en el endotelio de vasos, sobre todo pulmonares, en angiotensina II (AII). La AII, potente vasoconstrictor, produce retención de H_2O y Na^+ por dos mecanismos:

- Vasoconstricción intrarrenal, lo que disminuye la presión intraglomerular, causando una disminución de la filtración renal, con la consecuente disminución de diuresis y natriuresis.
- Estimulación de la secreción de aldosterona por la corteza suprarrenal, la cual retiene Na^+ y H_2O y excreta potasio (K^+). (Guyton y Hall, 1996) Sobre la aldosterona hablaremos más adelante en el punto 5.2.

Adicionalmente, se ha descrito que tanto la angiotensina II circulante como la que se produce de forma local en las paredes de los vasos sanguíneos, mediante un efecto endocrino y paracrino respectivamente, actúa sobre las células del músculo liso, teniendo efectos a largo plazo sobre la proliferación de músculo liso y remodelado vascular, de importancia en la patogenia de la hipertensión arterial (Weir y Dzau, 1999; Mehta y Griendling 2007)

5. HIPERTENSIÓN ARTERIAL. PAPEL DE LA ALDOSTERONA

Existe hipertensión arterial (HTA) cuando los valores de presión arterial sistólica (PAS) son mayores a 140 mm de Hg y/o los de presión arterial diastólica (PAD) mayores a 90 mm de Hg. Actualmente, se definen una serie de estadios de la HTA que están recogidos en la tabla 1. Se trata de una patología muy prevalente y con una gran morbimortalidad, al ser factor de riesgo independiente para enfermedades cardiovasculares. En España, se calcula que la prevalencia de la HTA es en la población general de un 35%. La prevalencia aumenta en varones y con la edad. Sin embargo, se calcula que sólo el 65% de los hipertensos tienen conocimiento de su enfermedad, y que de estos, sólo se controla el 25%, por lo que el control total del a HTA es de aproximadamente un 15% (Banegas y col, 2002). De esta manera, el no controlar la HTA produce un aumento del riesgo de infarto de miocardio, insuficiencia cardiaca, accidentes cerebrovasculares isquémicos y hemorrágicos, retinopatía e insuficiencia renal, entre otros (Chobanian y col, 2003)

	NORMOTENSIÓN			HTA		
CATEGORÍA	**Óptima**	**Normal**	**Norm-alta**	**Grado 1**	**Grado 2**	**Grado 3**
PAS, mm Hg	<120	120-129	130-139	140-159	160-179	≥ 180
	y	**o**	**o**	**o**	**o**	**o**
PAD mm Hg	<80	80-84	85-89	90-99	100-109	≥ 110

Tabla 1: Clasificación de la HTA. Fuentes: ESH/ESC Gudelines, 2003; Chobanian y col, 2003

En la fisiopatología de la HTA influyen multiples y complejos factores. Podemos dividir la HTA en secundaria, que es aquella en la que se identifica alguna causa y constituye menos de un 10% de los casos (hiperaldosteronismo primario, hipertensión renovascular, síndrome de Cussing, feocromocitoma, estenosis de aorta abdominal...); y primaria o esencial, que es objeto de nuestro y constituye más del 90% de los casos. La variedad y complejidad de sistemas implicados en la patogenia de la HTA hace su estudio muy complicado, ya que no puede atribuirse la responsabilidad absoluta de su etiología a ninguno de ellos (Kumar y col, 1997).

5.1 Fisiopatología de la HTA esencial

5.1.1 *Factores genéticos*

Si bien en humanos hay formas raras de HTA que afectan a un solo gen, este no parece ser el caso de la HTA esencial, aunque si es cierto que se ha visto cierta agregación familiar entre hipertensos, llegando a la conclusión de que, de existir influencia genética en la HTA, esta es poligénica (Braunwald y col, 2002, vol I).

Se han postulado la existencia de alteraciones de membrana celular en hasta el 50% de pacientes con HTA, en los cuales se produciría un aumento de la acumulación de Ca^{2+} intracelular, que en el caso de las células musculares lisas vasculares produciría

una hiperreactividad a los agentes vasoconstrictores. Sin embargo, no está claro si estas anomalías son primarias o adquiridas ante estímulos ambientales (Braunwald y col, 2002, vol I)

Un defecto en la excreción de Na^+ también ha sido postulado, de tal manera que se acumularía Na^+ y H_2O, aumentando el gasto cardiaco. Lo que en principio supone una respuesta fisiológica, al perpetuarse tiene consecuencias deletéreas sobre la función cardiaca y vascular, agravando la HTA y produciendo así un círculo vicioso. (Kumar y col, 1997)

5.1.2 *Factores ambientales*

El hecho de que existan diferencias en pacientes chinos y negros africanos en cuanto a niveles de HTA según vivan en sus zonas de origen (menor riesgo cardiovascular) o en países más desarrollados (mayor riesgo cardiovascular); o entre distintos estratos sociales, indica una clara influencia de factores ambientales en la HTA (Kumar y col, 1997).

Entre estos factores ambientales, aunque existe controversia, el consumo de sal se ha relacionado con la HTA desde hace años. Sin embargo, sólo el 50-60% de pacientes son sensibles a la sal, es decir, una ingesta excesiva de sal aumenta sus niveles de PA. Las causas de estas diferencias no están claras (Braunwald y col, 2002, vol I). Cabe destacar que en animales hipertensos sensibles a la sal que son alimentados con sales sódicas sin cloruro no se produce un aumento significativo de la PA, por lo que parece que el ion cloruro es igual de importante que el Na^+ en la génesis y mantenimiento de la HTA (Braunwald y col, 2002, vol I). También se ha descrito, en estudios epidemiológicos, un aumento de la incidencia de HTA asociado a un consumo bajo de calcio (Wimalawansa y col, 1995).

Obesidad, alcohol, sedentarismo y estrés son otros factores ambientales asociados a la aparición de HTA.

La resistencia a la insulina es un defecto común en la diabetes mellitus tipo 2 y la obesidad. Por otra parte, son cada vez más los datos que apoyan un papel de esta resistencia a la insulina en la génesis de la HTA, independientemente de que el paciente sea o no diabético y/u obeso. Así, la insulina produce, entre otros efectos, hipertrofia del músculo liso vascular y alteraciones de la permeabilidad del Ca^{2+} en las membranas celulares (Braunwald y col, 2002, vol I).

La conjunción de factores genéticos e influencias ambientales darían como resultado un desajuste en el control del GC y de las RP, lo que alteraría la regulación normal de la PA. Esto conllevaría que las respuestas fisiológicas que hemos comentado anteriormente, entre ellas la activación del sistema renina-angiotensina-aldosterona, pasen de ser respuestas adaptativas a respuestas patológicas, desarrollándose la HTA y sus complicaciones. La participación de este sistema en la fisiopatología de la HTA ha sido ampliamente estudiada, de tal forma que hoy se le atribuye un papel importante (pero no único). Los efectos de la AII y de la aldosterona van más allá de la regulación del equilibrio de Na^{2+} y H_2O, produciendo efectos locales sobre el corazón y los vasos, tales como fibrosis, hipertrofia muscular, daño endotelial, sensibilización a vasoconstrictores y remodelado vascular (Mehta y Griendling, 2007). Puesto que la aldosterona es uno de los objetos centrales de este trabajo, vamos a centrarnos en sus propiedades y en su participación en la HTA.

5.2 Aldosterona e HTA

Las hormonas esteroideas juegan un papel importante en la génesis de la HTA. Así, en mujeres hay una menor frecuencia y mejor control de la HTA que en hombres hasta la menopausia, a partir de la cual se igualan a la de los hombres (Braunwald y col,

2002, vol I); los anticonceptivos orales aumentan el riesgo de HTA (Rangarajan y, Kochar, 2000). Además, el exceso de glucocorticoides exógenos o endógenos produce HTA (Magiakou y col, 2006)

Por otra parte, la aldosterona, por su importante papel en la regulación a largo plazo de la PA, ha sido ampliamente estudiada en la fisiopatología de la HTA.

5.2.1 *Mecanismos de acción de la aldosterona*

La aldosterona es una hormona esteroidea sintetizada a partir del colesterol en la capa más superficial de la corteza suprarrenal, la capa glomerulosa, siendo el principal mineralocorticoide circulante (Florez y col, 1997). La figura 1 muestra los pasos en la síntesis de la aldosterona de forma esquemática. Sus funciones principales son la retención de H_2O y Na^+ y la excreción de potasio (K^+) e hidrogeniones (H^+) a nivel renal.

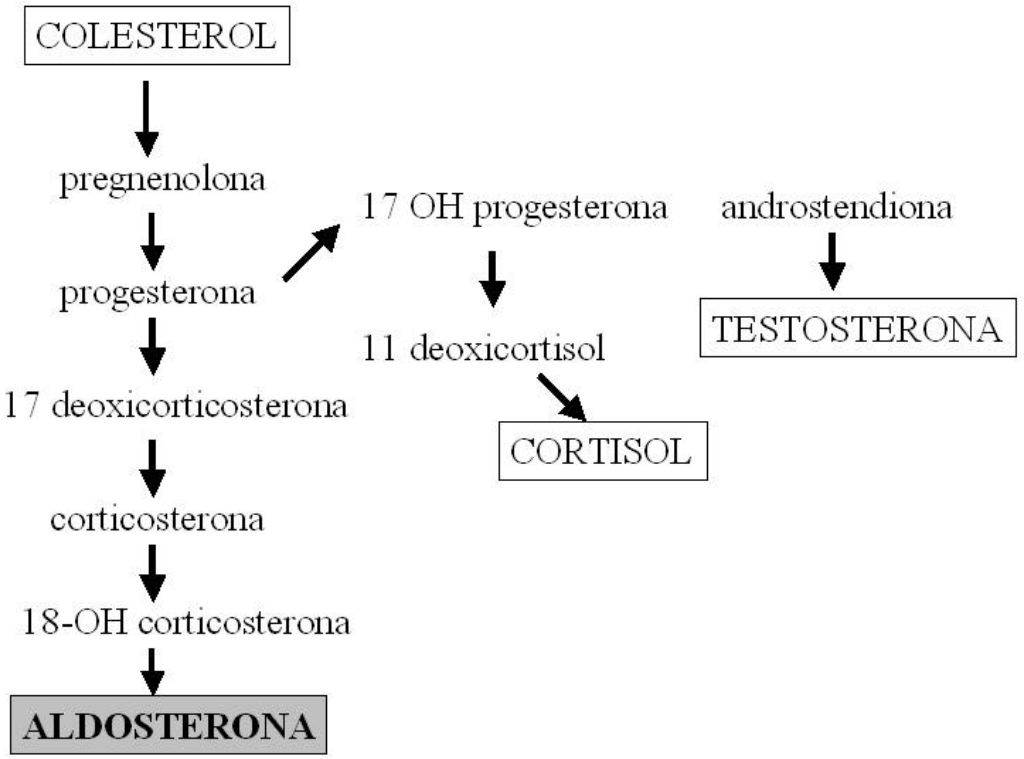

Figura 1: Síntesis de la aldosterona y de los otros esteroides de la corteza suprarrenal

La AII activa a las células de la capa glomerulosa de la corteza suprarrenal, aumentando la secreción de aldosterona. Ésta penetra en el citoplasma de las células del túbulo colector de la nefrona y se une a una proteína citoplasmática altamente específica, difundiendo al núcleo, en donde activa a su receptor mineralocorticoideo nuclear, aumentando la transcripción de proteínas necesarias para la expresión del intercambiador Na^+-K^+-H^+. Este intercambiador hace que se reabsorba Na^+ al espacio extracelular y se expulsen H^+ y K^+ a la orina. La reabsorción de Na^+ hace que, por aumento de la osmolaridad, se reabsorba H_2O, aumentando así el volumen extracelular y consecuentemente la PA (Guyton y Hall, 1996)

El hecho de reabsorber cantidades equivalentes de H_2O y Na^+ hace que se consiga aumentar la PA con una relación de ganancia H_2O y Na^+ casi cero, conociéndose este fenómeno como escape de la aldosterona. Esto explica que en el hiperaldosteronismo primario no se produzcan edemas a pesar de aumentar la PA (Guyton y Hall, 1996).

El aumento de la concentración de K^+ en el líquido extracelular es el otro estímulo principal de la secreción de aldosterona.

La aldosterona no sólo puede activar receptores mineralocorticoideos, sino que, a altas dosis, activa receptores glucocorticoideos (Balfagón y col, 2004; Farman y Rafestin-Oblin, 2001)

La aldosterona tiene un papel importante en la fisiopatología de la HTA por varios motivos:

- Síndromes que cursan con hipersecreción de aldosterona, tales como la hiperplasia suprarrenal bilateral o el síndrome de Conn, cursan con hipertensión arterial severa (Braunwald y col, 2002, vol I)

- La aldosterona retiene H_2O y Na^+, elevando la PA, lo que de forma prolongada puede contribuir, como ya hemos comentado, a la aparición de HTA
- La aldosterona tiene efectos vasculares y cardiacos deletéreos inedependientemente de la elevación de la PA, lo que contribuye al agravamiento de la HTA. Así, se han descrito aumento del remodelado vascular y aumento de fibrosis cardiaca (Rocha y Funder, 2002), además de un deterioro de la función endotelial (Blanco-Rivero y col, 2005) asociados a niveles altos de aldosterona.
- En pacientes normotensos pero con niveles de aldosterona superiores a la normalidad (sin llegar a ser hiperaldosteronismo primario) se ha visto un incremento de la frecuencia de HTA (Vasan y col, 2004)
- Fármacos inhibidores del receptor para aldosterona tales como la espironolactona o la eplerrenona se utilizan con éxito en el tratamiento de la HTA esencial y otras enfermedades cardiovasculares (Lahera y col 2006; Padilla y col, 2007)
- Por último, se ha descrito la presencia de concentraciones elevadas de aldosterona, superiores a las plasmáticas, en la pared vascular en HTA (Pasquale y col, 2000)

La figura 2 resume los aspectos de la fisiopatología de la HTA relatados hasta el momento.

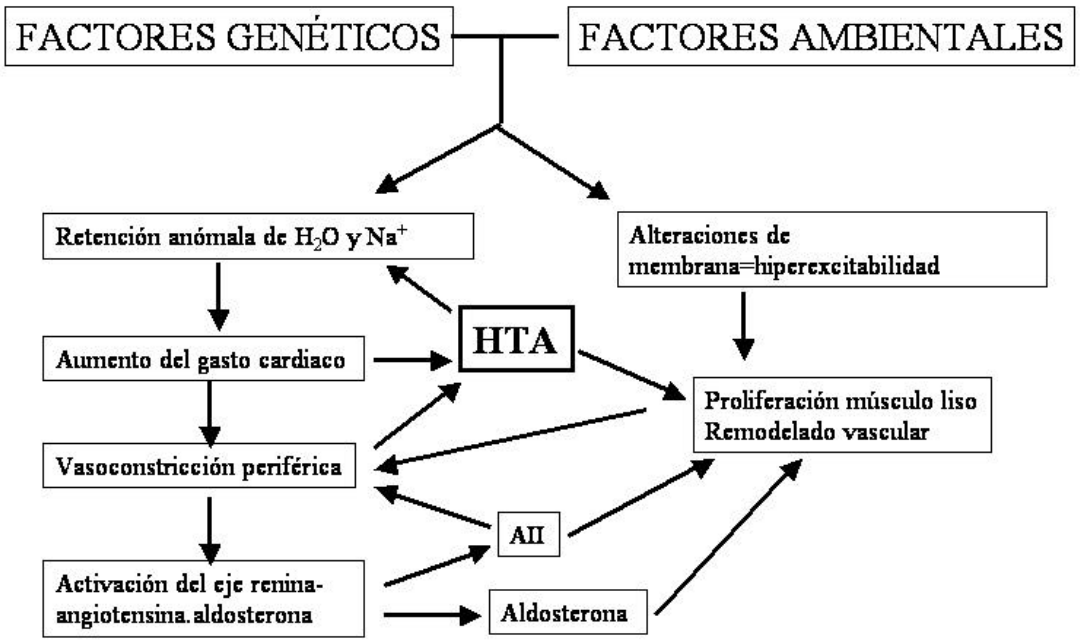

Figura 2: Representación esquemática de algunos de los aspectos de la fisiopatología de la HTA

6. CGRP

El CGRP es un neuropéptido de 37 aminoácidos ampliamente distribuido por el sistema nervioso central (SNC) y periférico y por el sistema cardiovascular. Es el vasodilatador endógeno más potente que existe y su participación en la fisiopatología de la HTA ha sido ampliamente estudiada. Una descripción detallada de su estructura, función, regulación, receptores, participación en la HTA en modelos animales y en humanos, así como de sus posibilidades terapéuticas en la HTA y enfermedades relacionadas, tales como la pre-eclampsia o el vasoespasmo posterior a la hemorragia subaracnoidea, estan recogidas en la **PUBLICACIÓN 1** de la presente tesis.

OBJETIVOS

El hecho de que el CGRP y la aldosterona tengan un papel importante en la fisiopatología de la HTA, nos lleva a preguntarnos si existe alguna relación entre ellos. En concreto, los objetivos de la presente tesis fueron:

1. Revisar la bibliografía para presentar, de forma resumida y actualizada, el papel que el CGRP tiene en la fisiopatología de la HTA así como sus posibiladades terapéuticas (**PUBLICACIÓN 1**)
2. Analizar el efecto de la aldosterona sobre la respuesta vasomotora inducida por estimulación eléctrica en arterias mesentéricas de ratas normotensas e hipertensas, analizando el papel del CGRP (**PUBLICACIÓN 2**)
3. Analizar el efecto de la aldosterona sobre la expresión de los componentes del receptor de CGRP, CL Receptor y RAMP1, en arterias mesentéricas de ratas normotensas e hipertensas (**PUBLICACIÓN 3**)
4. Analizar el efecto de la aldosterona sobre la expresión de los componentes del receptor de CGRP, CL Receptor y RAMP1, y si este mineralocorticoide modifica la respuesta vasomotora a CGRP en arterias cerebrales de ratas normotensas e hipertensas (**PUBLICACIÓN 4**)

RESUMEN PUBLICACIÓN 1

El péptido relacionado con el gen de la calcitonina (CGRP) es un neuropéptido de 37 aminoácidos y es el vasodilatador endógeno más potente que se conoce. La participación del CGRP en la hipertensión arterial y enfermedades relacionadas, tales como la preeclampsia o el vasoespasmo posterior a la hemorragia subaracnoidea, son algunos de los temas más estudiados de esta asustancia.

En esta revisión, resumimos los aspectos publicados sobre el CGRP en la fisiopatología de la hipertensión arterial en humanos y en modelos animales. También discutimos los efectos de la administración directa del CGRP en el tratamiento de la hipertensión y de fármacos antihipertensivos que aumentan la liberación o la respuesta del CGRP endógeno: inhibidores de la enzima convertidora de angiotensina, antagonistas del receptor de angiotensina II, beta-bloqueantes, sulfato de magnesio para la preeclampsia y la rutecarpina, así como las posibilidades de uso del CGRP en la terapia génica para la prevención del vasoespasmo posterior a la hemorragia subaracnoidea.

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Pathophysiology and therapeutic possibilities of calcitonin gene-related peptide in hypertension

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1. Introduction

In 1982 it was discovered that the calcitonin gene generated a different mRNA through an alternative splicing process (2) that produced a neuropeptide, the a-calcitonin gene-related peptide (aCGRP) (57). A second CGRP gene, independent of the a-calcitonin gene, was discovered in 1985 (1) and was named ßCGRP. The sensitive innervation of blood vessels possesses several neuropeptides. CGRP, which produces a potent vasodilatory response (6), is the most important and abundant of these neuropeptides (83). Later studies on the role of CGRP in different cardiovascular pathologies have high-lighted its role in hypertension, pulmonary hypertension, cardiac insufficiency, preeclampsia and Raynaud's phenomenon, as well as in subarachnoid haemorrhage (83). The objective of this review is to present an up-to-date overview of the role of CGRP in the pathophysiology of hypertension and to indicate its therapeutic possibilities in hypertension and related pathologies such as preeclampsia and subarachnoid hemorrhage.

1.1. Structure and function of CGRP in cardiovascular system

CGRP has been implicated in inflammatory processes, pain, wound healing, and it has a slight hypocalcemiant effect, inhibits gastric acid secretion as well as acting as a growth factor, etc (5, 83). However, it is mainly known for its potent cardiovascular effects.

The a and ßCGRP neuropeptides have 37 amino acids, which differ by 1 amino acid in rats and 3 in humans. These neuropeptides belong to the calcitonin peptide superfamily formed by calcitonin, adrenomedullin, amylin and CGRP (a and ß). All are

coded on chromosome 11, except for amylin which is coded on chromosome 12, and all share similar

structure and function (83). The calcitonin gene on chromosome 11 only gives rise to aCGRP on neurons through alternative splicing of its mRNA (2). The gene is expressed as calcitonin in the parafolicular cells of the thyroid in normal conditions, but, in medullar thyroidal carcinoma or in aged individuals, the gene is expressed as CGRP (45, 81). ßCGRP is translated by a distinct gene, also on chromosome 11, that does not code for calcitonin (1). The scarce participation of the ß form in pathophysiological processes has resulted in most of the existing studies (including the present review) focused only on

aCGRP.

CGRP is synthesized in the neuronal soma and stored by vesicles which transport

it to the axon terminal for release. Most CGRP is localised in the central nervous system (CNS), where it acts as a neurotransmitter (63), and in the cardiovascular system acting as a potent vasodilator (5, 6, 83). Nearly all vascular beds are innervated by fibers containing aCGRP (78), substance P (SP) (74) and neurokinine A (42) as cotransmitters for the sensory innervation. These fibers are sensitive to the action of capsaicin, which acts through type 1 vanilloid receptors (VR1), (13, 74, 83). The nerves originate in the dorsal root ganglia (DRG) of the thoracic column and Gasser's ganglion and the nerve endings are located between the adventitia tissue and the media, mainly in arteries (26, 58).

Several papers have reported that the local vasodilatory effect of the CGRP released by the vascular wall nerve endings is the one that participates in the regulation of peripherical resistence. Once released, CGRP acts through specific receptors, part is

taken up by the sensory endings (60), part is metabolised and the remainder is carried off in the blood flow (5, 83). The concentration of circulating CGRP is in the picomolar range (83) and, when injected intravenously CGRP effects have a rapid onset (83). The role of circulating CGRP is still unclear.

It has been reported that the sensitive innervation in the renal system releases CGRP which increases glomerular filtration and natriuresis by relaxing the afferent arteriole.

This neuropeptide is also relevant in controlling arterial pressure during pregnancy,

where it probably counteracts the increased volemia, since it has been seen to rise during pregnancy in humans (59, 65). Additionally its vasodilator effect is increased in pregnant rats by a mechanism associated with the increase in progesterone levels in these animals (18, 22, 83).

Cerebral blood vessels are widely innervated by CGRP-releasing neurons that produce a notable vasodilatation. This observation has awakened interest in the role of CGRP in pathologies like migraines and cerebrovascular accidents, and has given rise to new therapeutic possibilities (17, 30).

In the heart, CGRP increases heart beat and contractility and also produces coronary vasodilation (5, 83). Additionally, a dual role for CGRP as a growth factor in the vascular wall has been reported; it acts as a growth factor in human umbilical vein endothelial cells

(27), but inhibits vascular smooth muscle growth (11, 55). This suggests that CGRP, as well as its vasodilator effect, may have other beneficial cardiovascular effects, such as the control of intimal proliferation in atherogenesis (83).

1.2. CGRP regulation and its receptors

In the rat mesenteric artery, CGRP acts as a vasodilator neurotransmitter and the evidence indicates that CGRP itself regulates its own release through negative feedback stimulating presynaptic CGRP receptors (49). Furthermore, interaction between the perivascular adrenergic system and sensitive nerves was observed. Apparently noradrenaline (NA) release from sympathetic nerves produces an inhibition in CGRP release through the activation of presynaptic a2 receptors, as described earlier for neuronal NA release regulation (19), while CGRP would counteract the effect of noradrenaline (NA) by producing vasodilation (35, 36, 38) In adition, an uptake by the capsaicin sensitive nerve has been reported (60).

CGRP production is positively regulated by neuronal growth factor (NGF) in DRG (5). It has been reported that glucocorticoids decrease the CGRP content in DRG (64) and attenuate the stimulation of CGRP production by NGF in DRG (66). On the other hand, female sex steroids seem to increase the CGRP content in DRG from rats (41).

CGRP acts through the activation of specific receptors. Calcitonin superfamily peptide receptors are composed of three subunits. The first subunit corresponds to a G-protein coupled receptor (GPCR) that can be either the calcitonin receptor (CT) or the calcitonin-like receptor (CL receptor, historically called Calcitonin Receptor Like Receptor, CRLR (53). The second subunit is a receptor activity modifying protein (RAMP1, RAMP2 or RAMP3) (53). The third subunit, the receptor component protein (RCP) is required for G-protein-coupled signal transduction (54). The interaction between CL receptor and RAMP1 is necessary to constitute a functional CGRP receptor, while other combinations result in receptors for other peptides of the same family (34). The mechanism by which RAMP1 makes CL receptor specific for CGRP

instead of other peptides of the calcitonin superfamily apparently results from the glycosylation of some specific amino acids of the CL receptor. When another RAMP, such as 2 or 3, glycosylates the CL receptor in another position, it loses its specificity for CGRP (33). This has relevant pathophysiological consequences since the expression of one or another combination changes the effects on a given tissue of each of the peptides.

Classically, two types of CGRP receptors have been described, 1 and 2, although the existence of the second receptor is still controversial (53). The pharmacological difference between the two receptor types is that the effects of type 1 receptors are blocked by the 8-37 fragment of aCGRP, while blocking type 2 receptor's effects requires higher concentrations of the antagonist (53, 83). On the other hand, some authors recommend that the CGRP receptor (combination of CL receptor-RAMP1) be named CGRPA to distinguish it from another that has recently been observed in the cerebellum and spinal cord, the CGRP-B, whose components are not yet clearly defined (7). The present review only refers to CGRP-A (CGRP receptor through the text) because this is the major form detected and evaluated in cardiovascular system (Fig. 1). There are CGRP receptors in the endothelium and vascular smooth muscle of most vascular beds studied (78, 83).

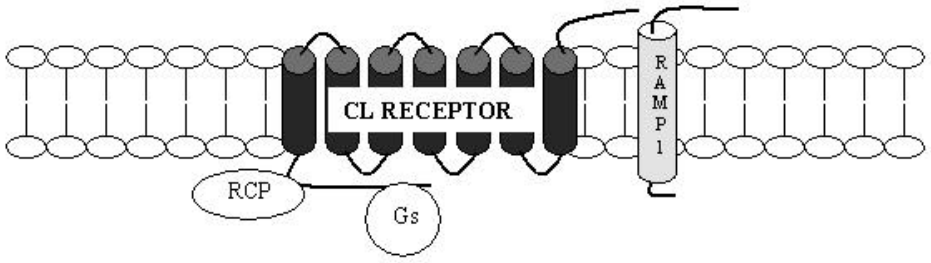

Fig. 1. Schematic representation of the components of the CGRP receptor.

The increase of cyclic adenosine monophosphate (cAMP) through protein G coupling to CGRP receptors is the main activation pathway of CGRP in the cells (5, 83). The so-called “endothelium dependent” response in the vascular wall consists in an increase in nitric oxide (NO) production through protein kinase A (PKA) activation (83). The activation of CGRP receptors located in the smooth muscle cells produces vasodilation by opening potassium ATP- dependent channels (KATP) (48, 78, 79, 83). Cell proliferation

also seems to be mediated by the increase in intracellular cAMP (77). Other mechanisms such as increased production of cAMP independent NO, of an endothelial relaxing factor (EDRF) other than NO (83) or of prostacyclin (PGI2) (12) have also been reported. Whatever the pathway, the end result is a decrease in intra-cytoplasmatic Ca2+ concentration , which induces relaxation in vascular smooth muscle. This decrease in Ca2+ is the result of decreased Ca2+ release from the intracellular reservoirs (39) (Fig. 2).

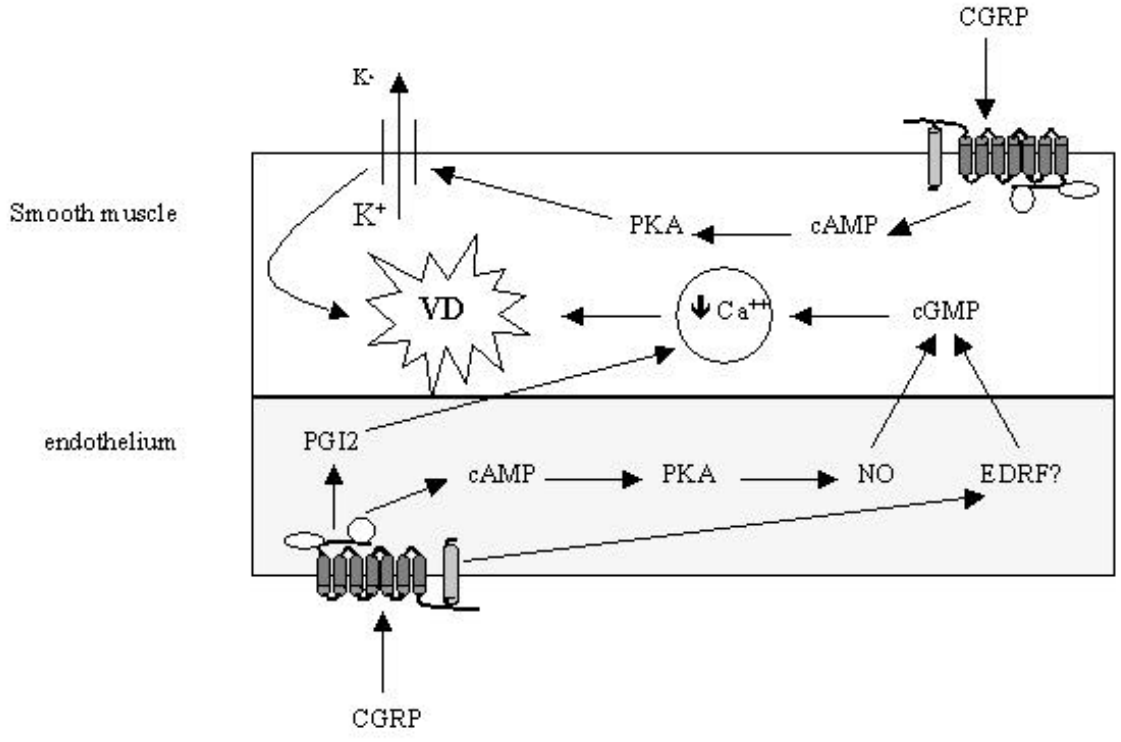

Fig. 2. Schematic representation of the intercellular signaling pathways for the CGRP.

CGRP receptor expression is modulated by different steroid hormones (20). In cultured smooth muscle cells from human coronary arteries, dexametasone increases CL receptor and RAMP1 mRNA expression (20). In rat mesenteric artery (85) and in mouse uterine smooth muscle (46, 47), female sex steroid hormones regulate CGRP receptor components. The increased sensitivity to CGRP observed in different vascular beds during pregnancy has been attributed to these processes (22, 23, 47, 85). Our group has recently

observed that aldosterone increases the vasodilatory response to CGRP in rat mesenteric arteries from spontaneously hypertensive rats (SHR) (3) and that this aldosterone effect is mediated by an upregulation of RAMP1 (43)

2. CGRP in hypertension

2.1. Experimental models of hypertension

In contrast to normotensive rats, decreases in plasma CGRP concentration (84), neuronal CGRP age related expression (38, 87) and the vasodilator response to spinal cord stimulation mediated by CGRP- containing vasodilator nerves (38) have been reported to spontaneously hypertensive rats (SHR). On the other hand, since CGRPergic nerves inhibits adrenergic neuronal activity (35), the decreased function of CGRPergic nerves in SHR enhances the adrenergic vasoconstriction (38). Taken together, these results support the hypothesis that a malfunction of CGRP nerves involved in peripheral vascular resistance control may play an important role in the pathogenesis and maintenance of hypertension in SHR. Further, a higher sensitivity to CGRP has been described in different vascular beds in the SHR, and this may act as a counterregulatory mechanism (71, 86).

The CGRP mRNA accumulation in DRG is higher in deocorticosterone (DOC)-salt rats than in normotensive rats. Treatment with CGRP 8-37 (a CGRP receptor antagonist) further increases mean arterial pressure, in a dose dependent manner, only in the DOC salt

rats. Therefore, it seems that CGRP plays an important haemodynamic role and has a counter-regulatory effect in these rats (67, 69). In contrast, in subtotal nephrectomy (SN) salt hypertensive rats, CGRP mRNA accumulation in DRG was similar to that found in normotensive rats and treatment with CGRP 8-37 produced a greater dose-dependent increase in arterial pressure than it did in normotensive rats. These results suggest that sensitivity to CGRP is enhanced in SN salt hypertensive rats (68, 70).

By activating the VR1 vanilloid receptor (13), capsaicin causes CGRP release from capsaicin-sensitive sensory nerve (83). Injection of capsaicin in normotensive rats does not modify their arterial pressure although the CGRP content in DRG decreases. When the injection is accompanied by a sodium overload or some alteration in renal function, there is

an increase in blood pressure (13, 74). These data seem to indicate that CGRP is important for arterial pressure regulation, in particularly through its effect on renal function. On the other hand, aCGRP Knock Out (KO) mice develop hypertension under physiological conditions without undergoing renal function alteration or receiving a sodium-rich diet (21, 50). In these animals, aCGRP mRNA is absent from the DRG while ßCGRP mRNA levels

were reduced two-fold, and so it would seem that aCGRP has a role in the long-term regulation of basal blood pressure. What is more, as well as eliminating a vasodilator (21), CGRP KO-induced hypertension could be also a result of eliminating the counter-regulatory effect of CGRP in the noradrenergic innervation (50).

Animal models that attempt to imitate preeclampsia with N(G)-nitro-L-arginine methyl ester (L-NAME) administration to pregnant rats have reported that coadministration

with CGRP can prevent the gestational hypertension and decrease fetal mortality (24). Moreover, thevasodilator effects of CGRP have been shown to be progesterone dependent (25). However, CGRP mRNA and peptide levels in DRG were not different between L-NAME treated and control rats (78). These data indicate that in this hypertension model, CGRP plays a counter-regulatory role, apparently due to a higher sensitivity to its vasodilator effects. These results are reflected in Fig. 3 and indicate that CGRP has different actions depending on the model of the hypertension studied.

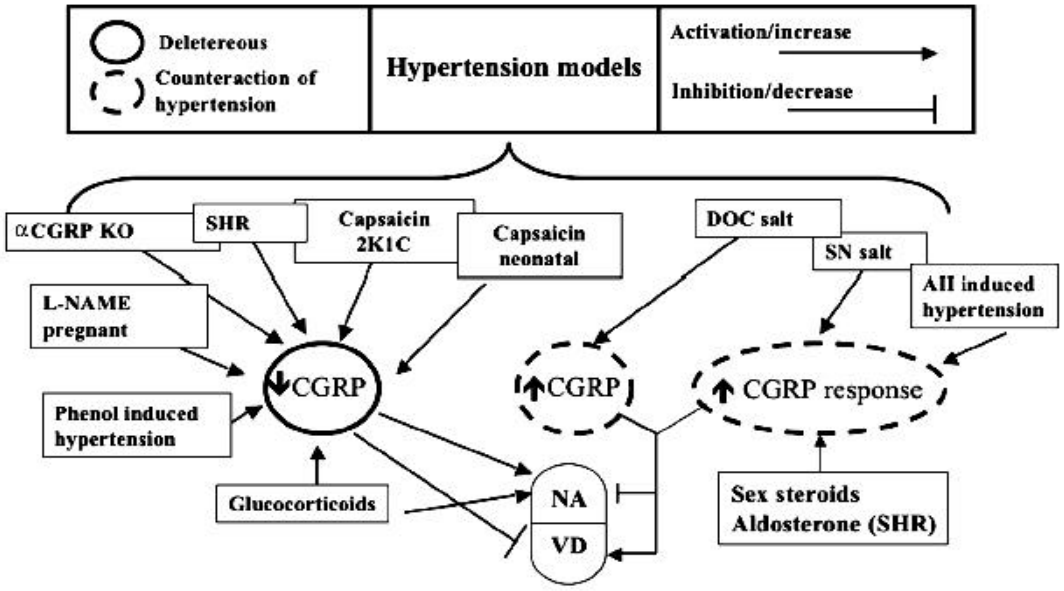

Fig. 3. The different roles for CGRP in the pathophysiology of hypertension in different animal models.

2.2. Human studies

Contradictory data on plasma CGRP levels in hypertensive patients have been published for years. Increases (44), decreases (52), and no changes (62) in CGRP plasma levels have all been reported. Recent methodological advances seem to confirm that arterial high pressure is associated to an increase in CGRP concentrations in plasma (44); this would suggest that CGRP, which has an intense vasodilatory effect when parenterally administered (83), would have a counter-regulatory role. The variations in the observations among hypertensive patients make difficult to distinguish whether the phenomena they present are causes or effects.

On the other hand, CGRP participation in hypertension in human patients has been reported to depend on whether the patient is sensitive to salt (SS) or resistant to salt (SR) (88). Decreasing sodium consumption in SR patients does not decrease arterial

pressure and the accompanying increase in plasma CGRP and renin levels (88) suggests that the vasodilatory effect of CGRP is unable to compensate for the effects of renin on vascular resistence. Decreasing salt consumption in SS patients does not modify the level of either CGPR or renin in blood (88). These observations support the hypothesis of some interaction between the reninangiotensin axis and CGRP nerve function.

It has also been observed that oral calcium intake decreases arterial pressure and that this decrease is accompanied by an increase in CGRP (80, 82). This suggests that plasma calcium levels influence the synthesis and or release of CGRP, although it needs to be experimentally confirmed.

The above results seem to indicate that in certain human hypertension conditions CGRP plays a counter-regulatory role and emphasises the need to develop more sensitive and specific techniques to dectect CGRP as well as a need for greater homogeneity in patients study groups so as to obtain more conclusive results. The different roles of CGRP depending on the effect of treatment, organ damage and evolution of hypertension should be considered in future studies.

3. Therapeutic possibilities

The possible use of CGRP as a therapeutic weapon is limited by the impossibility of administering it orally (it requires uncomfortable and difficult parenteral administration), its short half life (10 minutes) and its cost (83). However it could be a life saver in emergencies such as subarachnoid haemorrhage or preeclampsia. The study of the effects of different anti-hypertensive drugs on CGRP levels, for instance the angiotensisn converting enzyme inhibitors (ACE inhibitors), selective antagonists for the angiotensin II

receptor or of beta blockers, and the design of new drugs that have the effect of potentiating the release or response of endogenous CGRP, such as rutaecarpine, are opening new possibilities.

3.1. Anti-hypertensive drugs

The reninangiotensin system might be involved in the reduced function of CGRPergic nerves in SHR and in Goldblatt hypertensive rats. Different studies provided evidence that:

- Angiotensin II inhibits CGRP release through prejunctional angiotensin II receptors in the mesenteric artery of SHR (37).
- Long-term treatment with angiotensin converting enzyme inhibitors (captopril, temocapril) or angiotensin receptor antagonist (losartan) restore the reduced vasodilator response produced by CGRP nerves (37), prevents the reduction of CGRPergic perivascular

  innervation (29) in the mesenteric artery and increases levels of CGRP mRNA of the DRG (34) in SHR.

• In SHR, long-term treatment with calcium antagonists (nicardipine, amlodipine, pranidipine), beta-blockers (propranolol, pindolol) or hydralazine (vasodilator) do not restore the CGRP nerves function (37).

• In Goldblatt hypertensive rats, long term treatment with perindopril (angiotensin converting enzyme inhibitor) or losartan (angiotensin II receptor antagonist) increase plasma concentration of CGRP and CGRP mRNA expression in DRG (56).

Capsinolol was the first beta-blocker in which a CGRP-releasing effect was seen, specifically in the heart through the activation of VR1 vanilloid receptors (8). Carvedilol, an effective betablocker that is widely used for hypertension and other cardiovascular pathologies, increases CGRP release by activating VR1 receptors, particularly in the renal vessels of SHR rats (51).

3.2. Rutaecarpine

Rutaecarpine is the most important of the three allcaloids isolated from *Evodia rutaecarpa* , also known as "Wu-Chu.-Yu" and traditionally used in China (10) for the treatment of hypertension. Several mechanisms for the vasodilation provoked by rutaecarpine have been described (increased NO release, intracellular calcium alterations, an activation of potassium channels or prostaglandin production (9, 14, 75, 76),

and they all seem to be secondary to the activation of CGRP release through the activation of VR1 receptors (31, 40). Additionally it has been reported that rutaecarpine treatment decreases arterial pressure, increases vascular CGRP release and CRGP content in DRG in a model of phenol-induced hypertension in rats (14).

3.3. CGRP in preeclampsia

The participation of CGRP in blood flow regulation during pregnancy when the flow is augmented (16, 65) seems to be mediated principally by sex steroids (22, 23). A decrease in this peptide has been reported in preeclampsia and it has been reported that magnesium sulphate (widely used to treat preeclampsia) raises the plasma CGRP in these patients (28). On the other hand, vasodilatory response to CGRP and CGRP receptor expression in human umbilical vessels are impaired in preeclamptic patients (15). These observation together with those seen in animal models (24, 25) (see the corresponding section), constitute strong evidence that CGRP is involved in preeclampsia pathophysiology and suggest that its administration could constitute a therapeutic weapon in these patients.

3.4. CGRP in vasospasm after subarachnoid haemorrhage

Experimental models of subarachnoid haemorrhage (SAH) produce an abrupt CGRP release that attempts to compensate for the effects of the vasospasm (5, 30). Reports on the usefulness of CGRP intravenous (i.v.) administration to patients with a SAH are contradictory. One study reported that the prognosis was improved if the patients received an i.v. dose of CGRP after surgery (32) while a subsequent multicenter study found no differences among the control and treated groups (4). Taken these results into account it has been suggested that intrathecal administration would produce a more effective response than an i.v. route since it would have fewer general effects and greater local effects (83).

Several experiments have focus in the possibility of using CGRP-based gene therapy to prevent the cerebral vasospasm after SAH (72). Using adenovirus-mediated

gene transfer of CGRP in vivo in rabbits in which experimental SAH is provoked, it has been reported that there is a prevention of basilar artery vasospasm when compared with those rabbits receiving placebo (adenovirus with ß-gal gene instead of CGRP gene) (73). Similar

results have been reported using experimental models of severe vasospasm in dogs (61). These encouraging results open the possibility of using CGRP-based gene therapy for humans in the nearly future.

4. CONCLUSIONS

CGRP is relevant to the regulation of homeostasis in the cardiovascular system and it seems to be involved in different types of hypertension. Its decrease could be implicated in the genesis of some types of hypertension, while its increase in release and/or response could be a mechanism to counteract high blood pressure.

The direct administration of CGRP (although quite limited) and the use of drugs that increase the release and/or vasodilatory response to CGRP offer new therapeutic perspectives for the treatment of hypertension and preeclampsia. Rutaecarpine is the most specific CGRP-based treatment among the anti-hypertensive drugs analyzed, but further investigations are needed to elucidate its role and possibilities. Gene therapy is an encouraging alternative to prevent the vasospasm that occurs after SAH in humans and, in

future, clinical trials based on this therapy could be designed.

The results discussed in the present work open the possibility of new therapeutic perspectives for the treatment of different types of arterial hypertension, including preeclamsia, and SAH vasospasm, since CGRP function is involved in their pathophysiology.

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RESUMEN PUBLICACIÓN 2

Objetivo: Analizamos el efecto de la aldosterona en la respuesta vasomotora inducida por estimulación eléctrica (EFS) en arterias mesentérica de ratas Wistar Kyoto (WKY) y espontáneamente hipertensas (SHR).

Resultados: La aldosterona (0.001-1μM) redujo la respuesta vasoconstrictora inducida por EFS de forma tiempo y dosis dependiente sólo en SHR. De esta forma, el resto de experimentos se realizó sólo en SHR. La aldosterona no afectó ni a la liberación de NA ni a su efecto. El efecto de la aldosterona no fue alterado por L-N^G-nitro-arginina-metil-ester (100μM), y fue abolida por capsaicina (0.5μM) y el bloqueante de receptor del péptido relacionado con el gen de la calcitonina (CGRP), CGRP 8-37 (0.5μM). El CGRP (0.1nM-0.1μM) produjo una relajación dosis dependiente, que fue aumentada por la aldosterona (1μM). La incubación con espironolactona (1μM), gliblencalmida (10μM), RU 486 (10μM), ODQ (10μM) o cicloheximida (10μM) disminuyo este aumento de la relajación a CGRP inducido por aldosterona, mientras que el SQ 22.536 no la modificó.

Conclusiones: la aldosterona disminuye la vasoconstricción inducida por EFS en arterias mesentéricas de SHR pero no en WKY. Este efecto está mediado por un aumento en la sensibilidad a CGRP, mediante activación de receptores glucocorticoideos. Por otra parte, en este efecto participa la activación de receptores de potasio ATP dependientes, así como un incremento de la síntesis de proteínas y cGMP.

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Aldosterone modulates neural vasomotor response in hypertension: role of calcitonin gene-related peptide

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1. Introduction

Vascular tone is determined by an equilibrium among several mechanisms in which innervation plays an important role. This regulation involves adrenergic, cholinergic, nitrergic, peptidergic and/or sensory innervations which are specific of the vascular bed considered [1]. Electrical field stimulation (EFS) is widely used to study the influence of neurotransmitters released by nervous endings on vasomotor response, which depends on the balance between relaxing and contracting agents. EFS applied to rat mesenteric arteries produces a vasoconstrictor response which is the integrated result of the release of different neurotransmitters, mainly noradrenaline (NA) from adrenergic nerve terminals, neuronal nitric oxide (NO) from nitrergic innervation and calcitonin gene-related peptide (CGRP) from sensory nerves [2, 3, 4 and 5].

Aldosterone is a mineralocorticoid which participates in electrolyte balance and plays an important physiological role in the long-term regulation of Na^+ and K^+ in the distal tubule and collecting duct [6, 7, 8 and 9]. In addition, aldosterone is now recognized to participate in a number of different pathophysiological effects in the cardiovascular system such as endothelial dysfunction, vascular smooth muscle cell hypertrophy and hyperplasia, fibrosis and inflammation, although the mechanisms underlying these effects are not well understood [10, 11, 12, 13, 14 and 15]. Aldosterone circulates at subnanomolar levels; however, cardiovascular tissues elaborate steroids, including aldosterone, with the result that is more concentrate in the vascular tissue than in the circulation [16]. Although aldosterone has lower affinity for glucocorticoids than mineralocorticoid receptors, the high concentration of aldosterone reached in vascular tissues activates glucocorticoid receptors [17].

Aldosterone has been associated with the pathogenesis of hypertension, although their significance and potential importance are not yet clear [18 and 19]. It has been reported that aldosterone participates in the regulation of vascular tone through a variety of mechanisms. In fact, aldosterone has been involved in the regulation of catecholamine release, as well as in NO synthesis and metabolism [10, 13, 14 and 20]. In addition, steroid hormones have been reported to modulate vascular sensory innervation, although the possible role of aldosterone has not been studied yet [4, 21, 22, 23 and 24]. Consequently, we hypothesized that aldosterone could modulate the neural regulation of vasomotor tone acting on neurotransmitters released from the different nervous endings and to participate in the alterations of vascular function in hypertension. Therefore, the aim of the present study was to analyze: (1) whether aldosterone affects vasomotor response induced by EFS, (2) its possible modification by hypertension, (3) the mechanisms underlying this modification, and (4) the possible involvement of glucocorticoid receptors.

2. Materials and methods

2.1. Experimental procedure

Male 6-month-old Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) (weighing 200–250 g) were used. After the sacrifice by CO_2 inhalation, the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS) at 4 °C. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85.23, revised in 1996).

2.2. Protocol 1

Five consecutive frequency-dependent curves performed in the same segment from WKY or SHR were similar (data not shown), ruling out any change in EFS response along the experimental period. EFS responses in the absence or presence of aldosterone (0.001–1 μM) were performed to evaluate the possible effects of this mineralocorticoid on neural control of vasomotor tone. To analyse a possible time-dependent effect, aldosterone was added to the bath at different incubation periods, 30, 105, 180 and 255 min before the frequency–response curves, and was maintained in the media until the end of the experiment.

2.3. Protocol 2

The possible participation of adrenergic, nitrergic or sensory innervation in the effect of 1 μM aldosterone on the responses to EFS was analysed. Since no effect of

aldosterone was observed in the response to EFS in segments from WKY, the following experiments were only performed in segments from SHR.

To study the role of adrenergic innervation on the effect of aldosterone on EFS, the response to exogenous NA or the release of tritiated noradrenaline from adrenergic endings was evaluated in mesenteric segments from SHR. To analyze the possible participation of nitrergic innervation in the effect of 1 μM aldosterone on EFS response, experiments were performed in the presence of 100 μM of N^G-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor. To study the participation of sensory innervation in the effect of aldosterone on the responses to EFS, the sensory neurotoxin capsaicin (0.5 μM) was added to the bath 60 min before the second frequency–response curve and maintained until the end of the experiments. Since CGRP is the main mediator of sensory innervation, similar experiments were performed in the presence of the CGRP antagonist 0.5 μM CGRP (8–37), which was added to the media 30 min before the second frequency response curve and was maintained until the end of the experiments.

The response to exogenous NA, the main mediator of EFS response, was performed in the presence or absence of L-NAME, capsaicin or CGRP (8–37) in order to rule out any effect of theses agents on this response.

2.4. Protocol 3

The effect of aldosterone on the vasodilator response to CGRP as well as the possible mechanisms involved was studied. The ability of CGRP (0.1 nM–0.1 μM) to induce relaxation in arteries from SHR was assessed in segments contracted beforehand with NA (1 μM). To determine the possible effect of aldosterone on CGRP vasodilator response, this mineralocorticoid was added to the media before

NA contraction at the dose and incubation time where the maximal effect was observed. To analyse if the modulatory effect of aldosterone on CGRP vasodilator response was mediated by mineralocorticoid receptor activation, curves in the presence of 1 μM spironolactone were also performed. To analyse if the modulatory effect of aldosterone on CGRP vasodilator response was mediated by glucocorticoid receptor activation, curves in the presence of 10 μM RU 486 were performed. Since ATP-sensitive potassium channels are involved in vasodilator response to CGRP, the ATP-sensitive potassium channel blocker glibenclamide (10 μM) was added to the bath 30 min before the addition of aldosterone. To analyse the possible role of cGMP or cAMP synthesis on the effect of aldosterone on CGRP vasodilator response, curves in the presence of ODQ (10 μM) or SQ 22,536 (100 μM), respectively, were performed. Finally, to analyze whether the effects of aldosterone involve protein synthesis, cycloheximide (10 μM) was added to the original KHS at the time of artery removal and was maintained until the end of the experiment.

2.5. Vascular reactivity

The method used for isometric tension recording has been described elsewhere [25 and 26]. Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances to avoid any action by different drugs on the endothelial cells that could lead to misinterpretation of results. The segments were subjected to a tension of 0.5 g that was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mM K^+ to check their functional integrity. The absence of vascular endothelium was proven by the inability of 10 μM acetylcholine to relax segments precontracted with 1 μM NA.

Frequency–response curves to EFS (1, 2, 4 and 8 Hz) were performed in segments from both strains in a consecutive manner. The parameters used for EFS were 200 mA, 0.3 ms, 1–8 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A rest period of at least 1 h was necessary to avoid desensitization between consecutive curves.

Concentration–response curves to NA (10 nM–10 μM) were performed in segments from both strains in a consecutive manner. A washout period of 1 h was necessary to avoid desensitization between consecutive curves.

Concentration–response curve to CGRP (0.1 nM–0.1 μM) were perform in segments from SHR, previously contracted with NA.

2.6. Tritiated NA release

Tritiated NA release experiments were performed in denuded mesenteric segments from SHR. Segments were incubated with (±)-[^{3}H]-NA (0.33 μM, 10 μCi/ml, sp. act. 10 Ci/mmol). Three electrical stimulation periods of 60 s (200 mA, 0.3 ms, 4 Hz) were applied to the arteries at 60-min intervals. To evaluate the effects 1 μM aldosterone on tritium release, the mineralocorticoid was added to the superfusion media 30 min before the second stimulation and was maintained until the end of the experiment. The stimulation-induced tritium release was calculated by subtracting the basal tritium release (b1, b2, b3) from that evoked by electrical stimulation (S1, S2 and S3). Thereafter, the ratios of the net tritium release between S2/S1 and S3/S1 were calculated to eliminate differences between arteries. The effect of aldosterone on the evoked tritium release was expressed as its effect on these ratios. The amount of radioactivity released was expressed in dpm/mg wet tissue.

2.7. Solutions and drugs

The composition of KHS was as follows (mM): NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03 (to prevent the oxidation of unstable substances). Drugs used were: NA hydrochloride, acetylcholine chloride, L-NAME hydrochloride, -aldosterone, capsaicin, glibenclamide, spironolactone, CGRP, CGRP (8–37), RU 486, ODQ, SQ 22,536 and cycloheximide (Sigma, St. Louis, MO, USA). (±)-[^{3}H]-NA hydrochloride was from New England Nuclear (Boston, MA, USA). Stock solutions (10 mM) of drugs were made in distilled water, except for noradrenaline, which was dissolved in a NaCl (0.9%)–ascorbic acid (0.01% w/v) solution, and spironolactone and aldosterone in ethanol. These solutions were kept at -20 °C and appropriate dilutions were made in KHS on the day of the experiment.

2.8. Statistical analysis

The responses elicited by EFS or NA were expressed as a percentage of the concentration induced by 75 mmol/l K^+. The relaxation caused by CGRP was expressed as a percentage of the contraction induced by 1 μM NA. Results are given as mean±S.E.M. of eight rats. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-measure analysis of variance (ANOVA) followed by Bonferroni's test, using the Graphpad Prism 3.0 program (Graphpad Software, CA, USA). *P* values less than 0.05 were considered significant.

3. Results

3.1. Protocol 1

The presence of aldosterone (1 μM), added 30 and 105 min before the second and third curve, respectively, significantly reduced responses in segments from SHR (Fig. 1A) but not in WKY (data not shown). This reduction was significantly greater at 105-min than at 30-min incubation period (Fig. 1A). Addition of aldosterone (0.1 μM) to the media 30 min before the second frequency–response curve did not modify the vasoconstrictor response to EFS in segments from both SHR (Fig. 1B) and WKY (data not shown). However, incubation of aldosterone (0.1 μM) for 105 min significantly reduced the response to EFS only in segments from SHR (Fig. 1B). Incubation by 180 min with a lower concentration (0.01 μM) of aldosterone reduced the vasoconstrictor response to EFS only in the 50% of the segments from SHR rats (Fig. 1C). However, no changes were observed at 30 and 105 min (Fig. 1C). Likewise, this concentration did not modify EFS responses in segments from WKY in any time (data not shown). Low concentration of aldosterone (0.001 μM), added to the media maintained until 255 min, did not modify the vasoconstrictor response to EFS in segments from SHR (Fig. 1D) and WKY (data not shown).

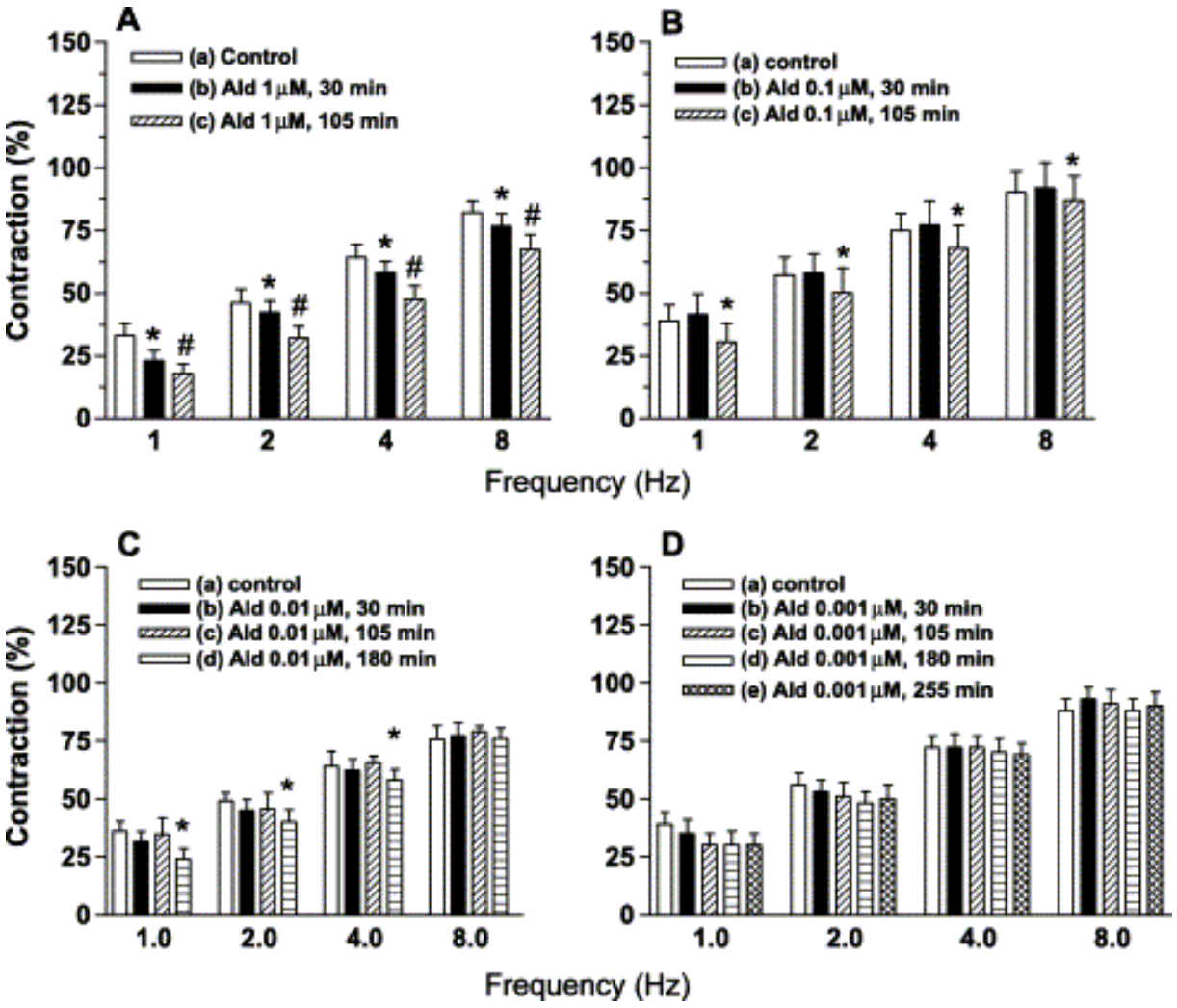

Fig. 1. Effect of aldosterone (Ald) [1 μM (A), 0.1 μM (B), 0.01 μM (C) and 0.001 μM (D)] at 30- to 255-min incubation period on frequency–response curves to EFS in SHR mesenteric artery segments. Results (mean±S.E.M.) from n=8 rats, each curve, are expressed as percent of previous tone with 75 mM KCl. $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. Ald 30 min.

3.2. Protocol 2

Three consecutive NA concentration-dependent curves performed in the same segment from WKY or SHR were similar (data not shown), ruling out any change in this response along the experimental period. The contractile responses elicited by NA in the presence of aldosterone (0.001-1μM), at the established time periods, remained unmodified in both strains (data not shown).

The contraction induced by EFS in segments from SHR was significantly increased by 100 µM L-NAME incubation (Fig. 2). Under these conditions, aldosterone (1 µM) added at the above mentioned time periods, reduced EFS-vasoconstriction in a comparable extent to that observed in absence of L-NAME (Fig. 2). L-NAME did not modify the vasoconstrictor response to exogenous NA (data not shown).

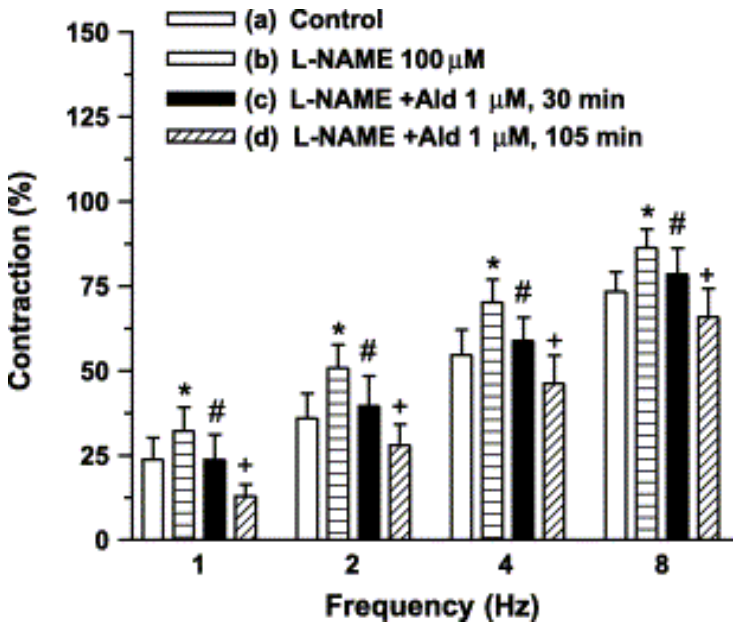

Fig. 2. Effect of aldosterone 1 µM at 30- and 105-min incubation period on frequency–response curves to EFS in SHR mesenteric artery segments in the presence of L-NAME (100 µM). Results (mean±S.E.M.) from n=8 rats, each curve, are expressed as percent of previous tone with 75 mM KCl. *p<0.05 vs. control; $^{\#}p$<0.05 vs. L-NAME; ^{+}p<0.05 vs. L-NAME+Ald.

The contraction induced by EFS was significantly increased in SHR segments by incubation with 0.5 µM capsaicin or 0.5 µM CGRP (8–37) (Fig. 3, right and left panels, respectively). Both agents abolished the effect of 1 µM aldosterone on EFS response

(Fig. 3). Either capsaicin or CGRP (8–37) did not modify the basal tone or vasoconstrictor response to exogenous NA (data not shown).

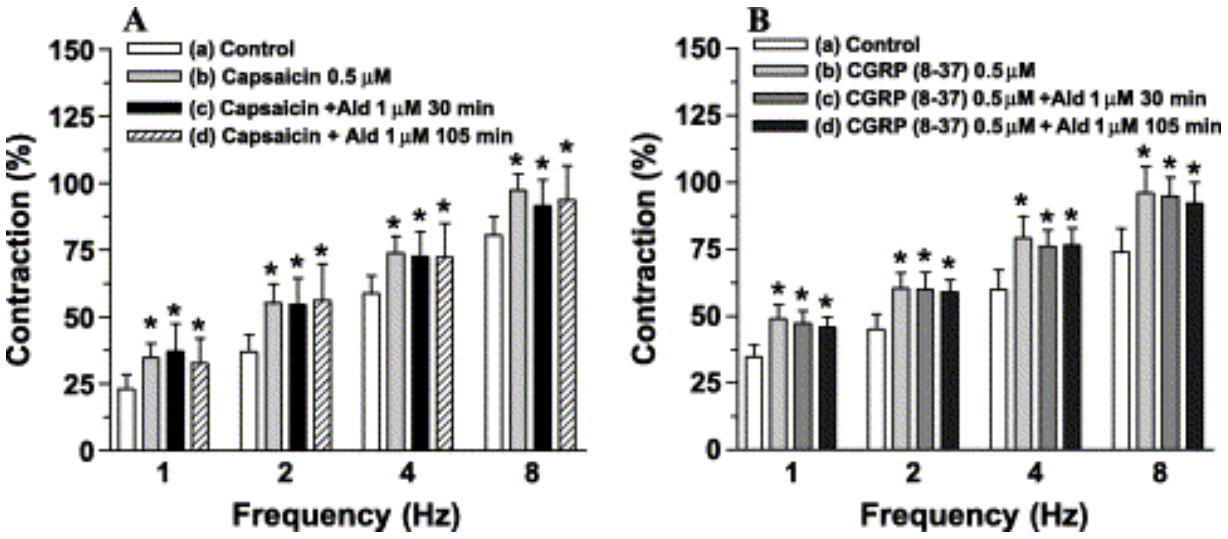

Fig. 3. Effect of aldosterone 1 μM at 30- and 105-min incubation period on frequency–response curves to EFS in SHR mesenteric artery segments in the presence of either capsaicin (0.5 μM) (A) or CGRP (8–37) (B). Results (mean±S.E.M.) from *n*=8 rats, each curve, are expressed as percent of previous tone with 75 mM KCl. **p*<0.05 vs. control.

Three consecutive EFS in mesenteric segments from SHR incubated with ^{3}H-NA induced similar tritium-overflow (S1=1403±81, S2=1361±90 and S3=1320±62 dpm/mg; S2/S1=0.97±0.7, S3/S1=0.94±0.8; *n*=8). Addition of 1 μM aldosterone 30 min before S2 and 105 min before S3 did not modify the EFS tritium overflow (S2/S1=0.98±0.12, S3/S1=0.89±0.10 dpm/mg; *n*=8). Addition of aldosterone did not affect basal tritium overflow (control: b1=114±7, b2=99±5 and b3=89±7; aldosterone: b1=111±9, b2=96±6 and b3=87±7 dpm/mg; *n*=8).

3.3. Protocol 3

In segments from SHR pre-contracted with NA (1 μM), CGRP induced a concentration-dependent relaxation, which was enhanced by aldosterone (1 μM, 105 min) (Fig. 4). The presence of spironolactone (1 μM), RU 486 (10 μM) significantly reduced the CGRP relaxation in the presence of aldosterone (Fig. 4). In addition, preincubation with ODQ (10 μM), glibenclamide (10 μM) or cycloheximide (10 μM) significantly reduced the CGRP-relaxation produced in the presence of aldosterone (Fig. 5). However, the presence of SQ 22,536 (10 μM) did not modify this response (Fig. 5).

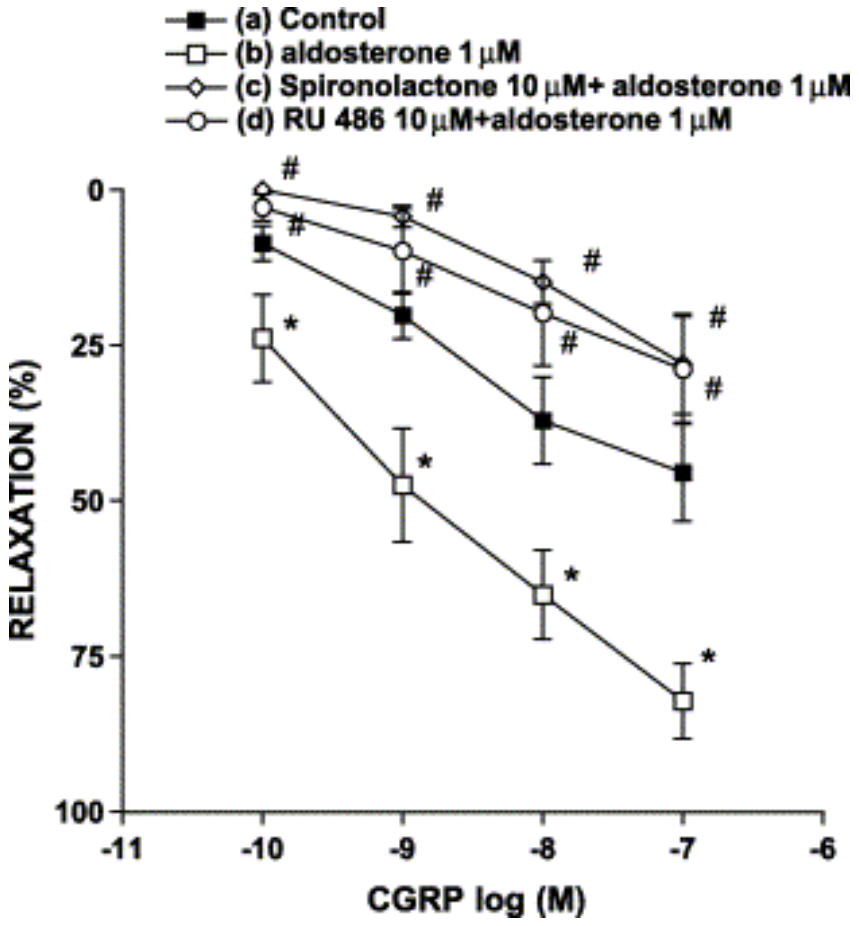

Fig. 4. Concentration-dependent relaxation to CGRP (a) in SHR mesenteric artery segments in the presence of aldosterone 1 μM (b), spironolactone (1 μM)+aldosterone (c), and RU 486 (10 μM)+aldosterone (d). Results (mean±S.E.M.) from *n*=8 rats, each

curve, are expressed as percent of previous contraction to NA. *p<0.05 vs. control; #p<0.05 vs. Ald.

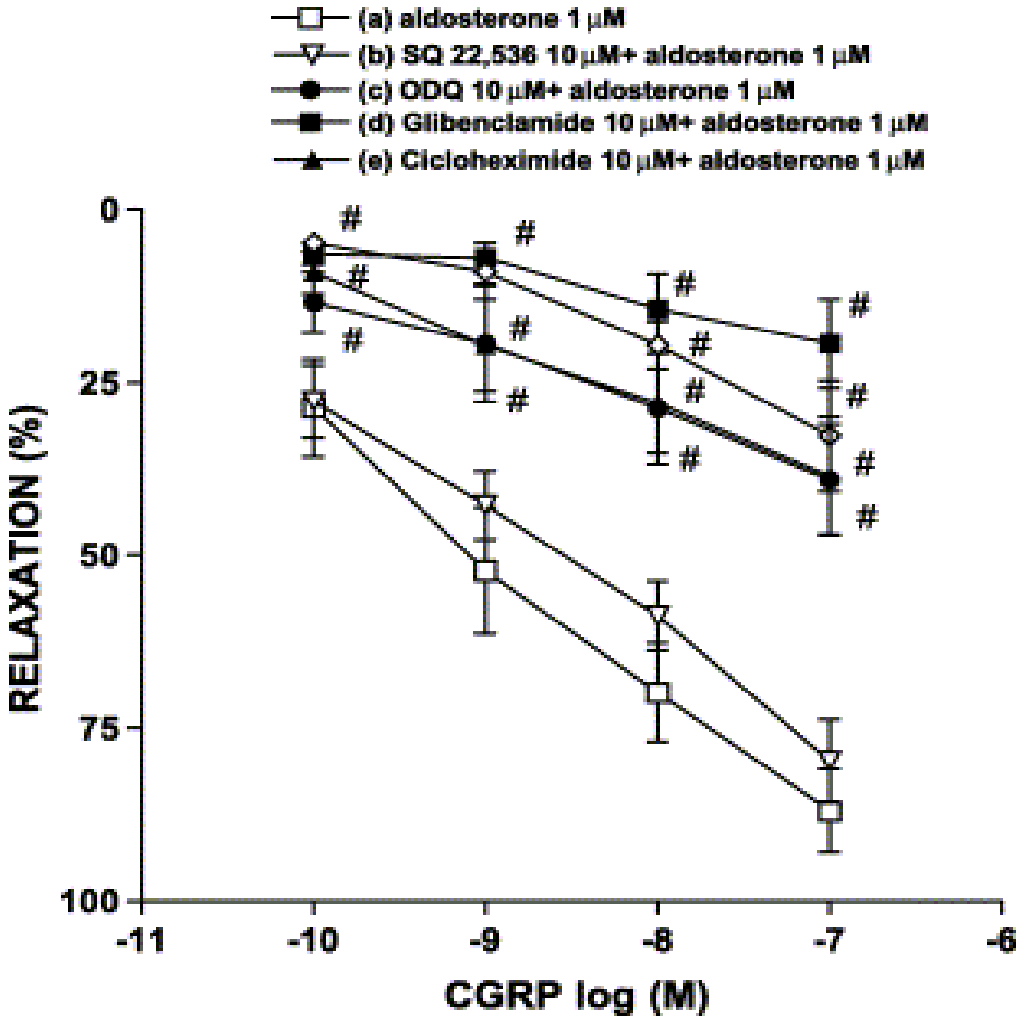

Fig. 5. Concentration-dependent relaxation to CGRP in SHR mesenteric artery segments in the presence of aldosterone 1 μM (a), SQ 22,536 (10 μM)+aldosterone (b), ODQ (10 μM)+aldosterone (c), glibenclamide (10 μM)+aldosterone (d), and cycloheximide (10 μM)+aldosterone (e). Results (mean±S.E.M.) from n=8 rats, each curve, are expressed as percent of previous contraction to NA. #p<0.05 vs. Ald.

4. Discussion

This study shows for the first time that in rat mesenteric arteries from SHR, but not from WKY, aldosterone decreased vasoconstrictor response elicited by EFS in a dose- and time-dependent manner.

It has been reported that aldosterone is able to affect vascular reactivity [27] Moreover, an interaction between aldosterone and the autonomic nervous system has been reported in several tissues [13]. On the other hand, we have previously demonstrated that EFS induces contractile responses in endothelium-denuded mesenteric arteries from WKY and SHR, and that these contractions appear to be mediated by the release of NA from adrenergic nerve terminals [5]. Consequently, the first objective of the present study was to analyse whether aldosterone was able to modify the vasoconstrictor response induced by EFS in mesenteric arteries and to evaluate the effect of hypertension on this response. Since previous reports have shown that aldosterone actions are dose- and time-dependent [10 and 28], four doses (0.001–1 μM) of aldosterone and four incubation periods (30–255 min) were studied. Frequency-dependent vasoconstriction remained unmodified in segments from WKY with the four doses of aldosterone used at the different incubation periods, indicating that aldosterone did not affect vasomotor response to EFS in normotensive rats. In contrast, in segments from SHR, aldosterone induced a decrease of EFS-vasoconstriction, which was dose- and time-dependent. These results indicate that aldosterone modulates vasomotor response to EFS only in hypertensive conditions. This effect of aldosterone in SHR could rely on specific modifications of the mechanisms involved in the neural control of vasomotor response associated with hypertension. It could be proposed that high aldosterone

concentrations at vascular level could exert a compensatory role on elevated vascular tone in hypertensive rats. Existence of other compensatory mechanisms in hypertensive conditions has been previously described. In fact, endothelin 1, angiotensin II and other vasoconstrictor factors which are overexpressed in hypertensive conditions are usually accompanied by a compensatory action of vasodilators, such as NO and prostacyclin [29, 30 and 31]. Furthermore, neural NO and CGRP release are enhanced in SHR when compared with normotensive rats [5].

Taking into account that NA is the main neurotransmitter released by EFS in the mesenteric artery, the observed effect of aldosterone in SHR could be due to two different mechanisms: a decrease in NA release from sympathetic nerve ending, and/or alteration in the sensitivity of vascular smooth muscle cells to NA [14, 27 and 32]. The fact that aldosterone (1 μM) modified neither EFS tritium release overflow nor vasoconstrictor response to exogenous NA indicates that aldosterone does not affect adrenergic innervation, and suggests that other mechanism should be involved in this effect. This is supported by the study showing that sympathetic nervous system is not modified in hypertension and primary aldosteronism [33].

It has been reported that aldosterone is able to modify the release of NO in different cell types [13, 20 and 34]. In addition, we have recently demonstrated that EFS also induces the release of NO from nitrergic nerves in mesenteric arteries from SHR rats [5]. Consequently, we analysed the possible participation of this innervation in the effect of aldosterone on EFS or exogenous NA responses, performing experiments in the presence of the NOS inhibitor L-NAME. However, the results showed that the presence of L-NAME did not modify the effect of aldosterone in either EFS or NA responses, ruling out any participation of the nitrergic innervation.

Since EFS response also involves sensory innervation [2, 3 and 5] and steroid hormones can modify the main mediator of this response, CGRP [4, 21, 22, 23 and 24], we also explore the possible participation of sensory innervation in the observed effect of aldosterone. The results showed that the presence of neurotoxin, capsaicin, abolished the decreased in EFS response induced by aldosterone, indicating that this effect involved sensory innervation. This participation was confirmed by the fact the presence of the CGRP antagonist, CGRP (8–37), also abolished the effect of aldosterone. In a previous work, using similar experimental conditions, we have described that CGRP participates in the response induced by EFS in mesenteric segments from SHR but not from WKY [5]. These results justify that aldosterone only has effect in arteries from SHR rats.

Several studies have shown that steroid hormones can interact with CGRP [4, 21, 22, 23 and 24]. Therefore, we explore the possibility that the observed effect of aldosterone might involve an enhancement of the sensitivity of smooth muscle to CGRP. Aldosterone significantly increased the vasodilator response to CGRP, suggesting that the effect of aldosterone on EFS response could be a consequence of an increased response to CGRP.

The mechanisms by which steroids could increase vasodilator response to CGRP are not totally understood. An increased release of endothelium-derived relaxing factors produced by steroids [4 and 35] has been proposed. However, the use of endothelium-denuded mesenteric segments rules out this possibility. In addition, an increase on CGRP receptors by sex steroids hormones treatment has been described in rat mesenteric arteries [24]. Since it has been reported that CGRP exerts its vasodilatory action through the activation of ATP-dependent potassium channels [4 and 36], experiments were performed in the presence of the ATP-

dependent potassium channel antagonist, glibenclamide. The enhancement of CGRP vasodilation produced by aldosterone was significantly decreased in the presence of glibenclamide, supporting the participation of ATP-dependent potassium channels in this effect. Since both cAMP and cGMP are involved in the depressor effects of CGRP [4], the effect of SQ 22,536 and ODQ, the inhibitors of adenylate cyclase and guanylate cyclase, respectively, on the CGRP vasodilation in the presence of aldosterone was analysed. The results obtained indicate the involvement of cGMP in this response but not of cAMP.

The observation that spironolactone, a mineralocorticoid receptor antagonist, reduced the enhancement of the vasodilator response to CGRP produced by aldosterone indicates that this effect could be mediated through the activation of mineralocorticoid receptor. However, the presence of the glucocorticoid receptor antagonist RU 486 reversed the effect of aldosterone on CGRP-relaxation, supporting the involvement of these type of receptors. The involvement of glucocorticoid receptors could be explained because aldosterone, at high concentrations, as used in our study, can activate glucocorticoid receptors. In addition, it should be mentioned that the inhibitory effect of spironolactone on the effect of aldosterone on CGRP-relaxation could also include the antagonism on glucocorticoid receptors produced by the elevated concentrations of this antagonist [37].

Finally, it has been reported that effects of aldosterone and glucocorticoids could involve genomic or non-genomic mechanisms [18, 28 and 38]. To explore this aspect, the effect of aldosterone on CGRP response was performed in the presence of the protein synthesis inhibitor cycloheximide. This protein synthesis

inhibitor blunted the effect of aldosterone on CGRP relaxation, supporting a genomic effect.

In summary, aldosterone was able to decrease the vasoconstrictor response to EFS in mesenteric arteries from SHR but not from WKY. This effect is mediated by an increased response to the sensory neurotransmitter CGRP, substantially, through glucocorticoid receptor activation. Furthermore, this effect is mediated by an increase of cGMP synthesis and ATP-dependent potassium channel activation.

Acknowledgements

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RESUMEN PUBLICACIÓN 3

Objetivo: Analizamos el efecto de la aldosterona sobre la relajación mediada por CGRP y el efecto de la aldosterona en la expresión de los componentes del receptor de CGRP: "Calcitonin-Like Receptor" (CL Receptor) y "Receptor Activity Modifying Protein 1" (RAMP1) en arterias mesentéricas de ratas WKY y SHR sin endotelio.

Resultados: CGRP 0.1nM-0.1μM indujo una relajación dosis dependiente que fue aumentada por aldosterona 1μM sólo en SHR. La incubación con RU 486 10μM redujo significativamente este efecto de la aldosterona en SHR. La expresión de CL Receptor no fue modificada por aldosterona, mientras que en SHR, la incubación con aldosterona 1μM y 0.1μM 120 minutos produjo un aumento de la expresión de RAMP1. Este aumento en la expresión de RAMP1 fue inhibido por el RU 486 10μM.

Conclusiones: La aldosterona, mediante activación de receptores glucocorticoideos, aumenta la actividad vasodilatadora del CGRP en arterias mesentéricas de SHR, efecto que parece estar mediado por un aumento en la expresión de RAMP1.

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Aldosterone increases RAMP1 expression in mesenteric arteries from spontaneously hypertensive rats

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1. Introduction

Vascular tone is determined by equilibrium among several mechanisms in which adrenergic, cholinergic, nitrergic, peptidergic and/or sensory innervation play an important role depending on the vascular bed studied [1] and [2]. In a recent study we demonstrated that aldosterone, by activating glucocorticoid receptors, decreases the vasoconstrictor response to electrical field stimulation (EFS) in endothelium-denuded mesenteric arteries from spontaneously hypertensive rats (SHR) by increasing the vasodilatory response to calcitonin gene-related peptide (CGRP) [3]. This decrease in the EFS vasoconstrictor response induced by aldosterone was dose and time dependent [3] and it was not observed in the normotensive model of Wistar Kyoto rats (WKY), suggesting that the increase in the vasodilator response to CGRP mediated by aldosterone is specific to hypertension [3].

CGRP is one of the most potent known vasodilators and it acts through specific receptors. It belongs to the superfamily of calcitonin peptides (calcitonin, CGRPa and ß, amylin and adrenomodulin) [4]. The CGRP receptor is one of the superfamily of G-protein coupled receptors (GPCRs) and is composed by three subunits: a seven transmembrane G protein coupled receptor called calcitonin receptor-like receptor (CL receptor), the single transmembrane-spanning receptor activity modifying protein 1 (RAMP1) [5] and the receptor component protein (RCP) [4]. The expression of RAMP1 in combination with CL receptor produces a specific and functional CGRP receptor, while the expression of the other RAMP family proteins RAMP2 or RAMP3 makes the receptor specific to other substances like adrenomodulin and amylin respectively [5].

It has been described that glucocorticoids increase the expression of RAMP1 and CL receptor in vascular smooth muscle cells [6]. Although we have not observed

modifications in functionality of the CGRP system by endogenous female [7] or endogenous male [8] sex steroids, other authors have suggested that sex steroids and pregnancy increase the vasodilatory response to CGRP in mesenteric arteries from rats [9] and that this effect is mediated by an increase in the mRNAs expression of CL receptor and RAMP1 [10].

Our first objective is to analyse whether aldosterone modifies the vasodilatory response to CGRP in WKY and SHR rats. Next, we analyse whether aldosterone-mediated increase of the vasodilatory response to CGRP in SHR rats [3] is caused by an increase of the expression of CGRP receptor components.

2. Materials and methods

2.1. Experimental procedure

Male 4-month-old Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) (weighing 200–250 g) were used. After sacrifice by CO_2 inhalation, the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS) at 4 °C. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85.23, revised in 1996).

2.2. Vascular reactivity

The effect of aldosterone on the vasodilator response to CGRP was studied. The method used for isometric tension recording has been described elsewhere [11] and [12]. Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances and to avoid any action by different drugs on the endothelial cells that could lead to misinterpretation of results. The segments were subjected to a tension of 0.5 g that was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mM K^+ to check their functional integrity. The absence of vascular endothelium was proven by the inability of 10 μM acetylcholine to relax segments precontracted with 1 μM noradrenaline (NA).

The ability of CGRP 0.1 nM–0.1 μM to induce relaxation in arteries from WKY and SHR was assessed in segments precontracted with NA 1 μM. To determine the possible effect of aldosterone on CGRP vasodilator response, this mineralocorticoid

was added to the media at a dose of 1 μM 120 min before NA contraction, the incubation time and dose were those of maximum effect of aldosterone in CGRP vasodilator response [3]. To determine the role of glucocorticoid receptors, a group of segments was pre-treated with the glucocorticoid receptors antagonist RU 486 at a dose of 10 μM 30 min before adding aldosterone. Another set of experiments was done adding RU 486 10 μM alone for 120 min alone to analyse the possibility of a direct effect of RU 486.

2.3. Western blot analysis

The segments from WKY and SHR mesenteric endothelium-denuded arteries were mounted in the same organ bath system in which vascular reactivity was studied. Experiments were done in the absence of endothelium. The absence of vascular endothelium was proven by the inability of 10 μM acetylcholine to relax segments precontracted with 1 μM NA. Segments from WKY were incubated 120 min with aldosterone 1 μM. Segments from SHR were divided into eight groups: control group, pre-treated with aldosterone 1 μM, 0.1 μM and 0.01 μM incubated for 60 or 120 min. To determine the role of glucocorticoid receptors, groups of segments were pre-treated with the glucocorticoid receptors antagonist RU 486 at a dose 10 μM for 30 min before adding aldosterone. Another group was incubated with RU 486 alone at a dose 10 μM for 120 min to analyse the effect of the antagonist alone on the expression of CL receptor and RAMP1.

Once incubation time had finished, segments were quickly frozen at - 80 °C. For Western blot analysis of CL receptor and RAMP1 protein expression, mesenteric arteries were homogenised in a boiling buffer composed of 1 mM sodium vanadate (a protease inhibitor), 1% SDS and 0.01 M pH 7.4 Tris–HCl. Homogenates containing

16.5 µg protein were electrophoretically separated on a 12% SDS-polyacrylamide gel (SDS-PAGE) and then transferred to polyvinyl difluoride membranes (Bio-Rad Immun-Blot®) overnight at 4 °C, 230 mA, using a Bio-Rad Mini Protean III system (Bio-Rad Laboratories, Hercules, CA, USA) containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS. Prestained SDS-PAGE broad range standards (Bio-Rad Laboratories) were used as molecular mass markers. The membrane was blocked for 3 h at room temperature in Tris-buffered–saline solution 100 mM (0.9% w/v NaCl, 0.1% SDS) with 5% non-fat powdered milk before being incubated overnight at 4 °C with rabbit antirat RAMP1 (1 : 250 dilution) or rabbit antirat CRLR (CL receptor) (1 : 1000 dilution) antibodies. After washing, the membrane was incubated with a 1 : 1000 dilution of anti-rabbit Immunoglobulin G antibody conjugated to horseradish peroxidase (Amersham). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International plc, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International Plc). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56). The same membrane was used to determine a-actin expression, and the content of the latter was used to correct CL receptor and RAMP1 expression in each sample, by means of a monoclonal antibody anti-a-actin (1 : 2000 dilution). A positive control (rat brain homogenate) for both RAMP1 and CL receptor was used in every experiment.

2.4. Solution and drugs

The composition of KHS was as follows (mM): NaCl 115, $CaCl_2$ 2.5, KCl 4.6, H_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03 (to prevent

the oxidation of unstable substances). Drugs used were: NA hydrochloride, acetylcholine chloride, d-aldosterone, CGRP, and RU 486 (Sigma; St. Louis, MO, U.S.A). Stock solutions (10 mM) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)–ascorbic acid (0.01% w/v) solution and aldosterone and RU 486, which was dissolved in ethanol. These solutions were kept at - 20 °C and appropriate dilutions were made in KHS on the day of the experiment. CRLR (CL receptor) polyclonal antibody (Alpha Diagnostic International, San Antonio, USA), RAMP1 polyclonal antibody (Santa Cruz Biotechnology Inc, Europe), a-actin mouse antirat antibody (Sigma; St. Louis, MO, U.S.A) and anti-rabbit horseradish peroxidase antibody (Amersham International plc, Little Chalfont, UK).

2.5. Statistical analysis

The relaxation caused by CGRP was expressed as a percentage of the contraction induced by 1 µM NA. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-measure analysis of variance (ANOVA) followed by Bonferroni's test.

For the Western blot analysis the values were represented as media ± SEM of the values normalized by actin. The statistical analysis was performed using the unpaired two-tailed *t* student test. Statistical analysis was made using the Graphpad Prism 3.0 program (Graphpad Software Inc. CA, USA). *p* values less than 0.05 were considered significant.

3. Results

3.1. Vascular reactivity

In segments from WKY and SHR pre-contracted with NA (1 μM) (mean of contraction 1031 ± 104 and 1069 ± 219 mg, respectively, $p > 0.05$), CGRP 0.1 nM–0.1 μM induced a concentration-dependent relaxation, which was enhanced by aldosterone 1 μM 120 min only in segments from SHR (Fig. 1 A and B). Aldosterone, whatever the dose and time of incubation, modified neither the basal tone of the arteries nor the contraction elicited by NA 1 μM. Presence of RU 486 10 μM, added to the bath 30 min before the aldosterone, significantly reduced the enhancement of CGRP-relaxation produced by the mineralocorticoid in SHR (Fig. 1B). RU 486 10 μM alone for 120 min did not modify the response to CGRP (Fig. 1B).

3.2. Western blot analysis

In segments from WKY rats two CL receptor bands were detected with molecular weights of 110 and 66 kDa and the RAMP1 band had a weight of 30 kDa. Aldosterone 1 μM at 120 min incubation did not modify the expression of any of the CL receptor bands or RAMP1 (Fig. 2 and Fig. 3).

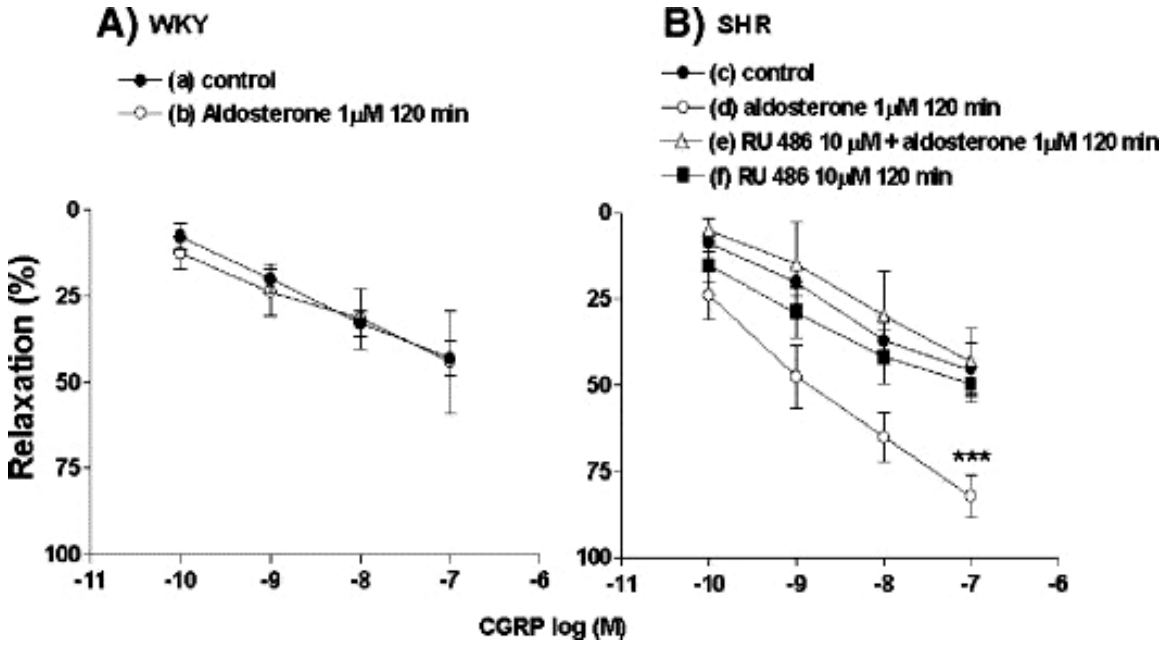

Fig. 1. Concentration-dependent relaxation to CGRP in WKY (A) and SHR (B) mesenteric artery segments. $n = 6$ animals per group. ***$p < 0.001$ vs. control.

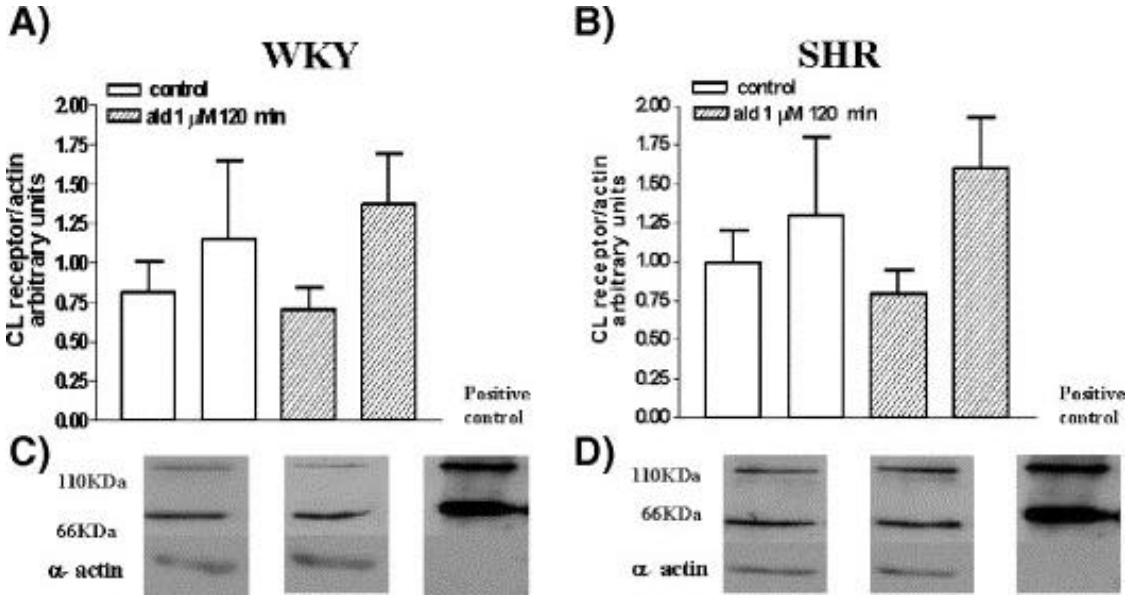

Fig. 2. Effect of aldosterone 1 μM on CL receptor 110 and 66 kDa band expression in WKY and SHR mesenteric artery segments. A) WKY and B) SHR: Densitometric analysis of 110 and 66 kDa bands from 5 animals per group. C) WKY and D) SHR: Representative immunoblot of mesenteric artery segments homogenate from WKY and SHR showing 110 and 66 kDa bands and a-actin from each group and the positive control.

In segments from SHR rats two bands were detected of CL receptor with molecular weights of 110 and 66 kDa and the RAMP1 band had a weight of 30 kDa. Aldosterone 1 µM at 120 min incubation did not modify the expression of any of the CL receptor bands (Fig. 2). Aldosterone 1 µM 120 min did significantly increase the expression of the 30 kDa RAMP1 band ($p < 0.05$) in SHR rats (Fig. 3). Presence of RU 486 10 µM, from 30 min before aldosterone addition, significantly reduced the enhancement of RAMP1 expression produced by the mineralocorticoid (Fig. 3). Aldosterone 0.1 µM significantly increased the expression of the 30 kDa RAMP1 band ($p < 0.05$) in SHR rats at 120 min (Fig. 4). Presence of RU 486 10 µM, from 30 min before aldosterone addition, significantly reduced the enhancement of RAMP1 expression produced by the mineralocorticoid (Fig. 4). Aldosterone 0.01 µM did not modify the RAMP1 expression in SHR (Fig. 4). The presence of RU 486 10 µM alone for 120 min did not modify RAMP1 basal expression (Fig. 3).

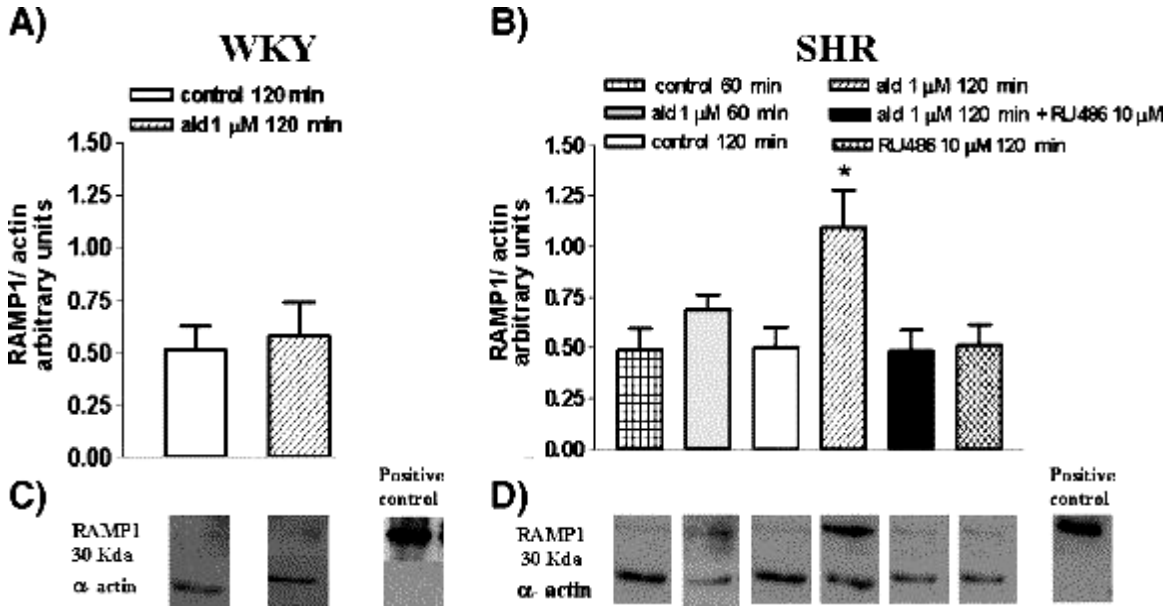

Fig. 3. Effect of aldosterone 1 µM alone and RU 486 10 µM + aldosterone 1 µM on RAMP1 30 kDa band (homodimer) expression in WKY and SHR mesenteric artery segments. A) WKY and B) SHR: Densitometric analysis of 30 kDa band from 5 animals per group. C) WKY and D) SHR: Representative immunoblot of mesenteric

artery segments homogenate from WKY and SHR showing 30 kDa band and a-actin from each group and positive control. *$p < 0.05$ vs. control 120 min.

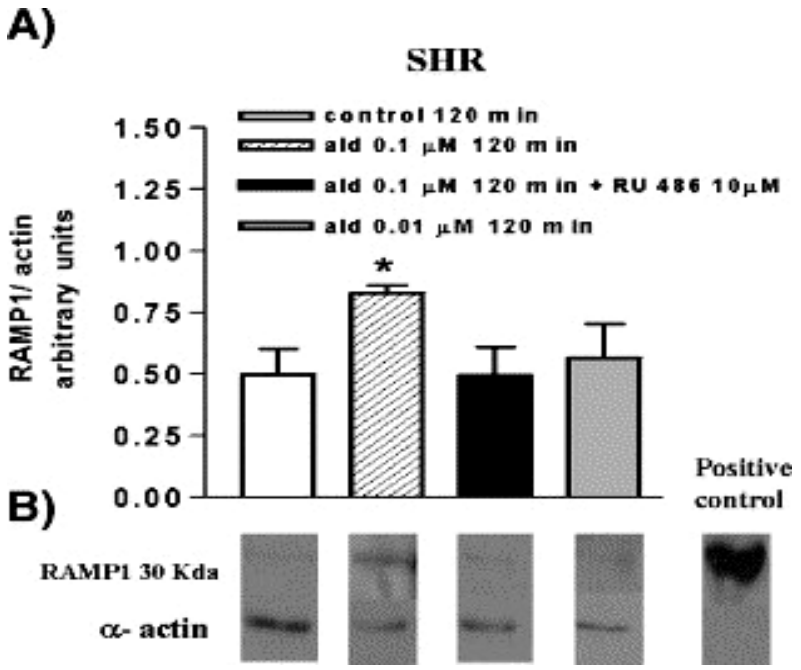

Fig. 4. Effect of aldosterone 0.1 µM, alone and RU 486 10 µM + aldosterone 0.1 µM and aldosterone 0.01 µM on RAMP1 30 kDa band (homodimer) expression in SHR mesenteric artery segments. A) Densitometric analysis of the 30 kDa band from 5 animals per group. B) Representative immunoblot of mesenteric artery segments homogenate from SHR showing 30 kDa band and a-actin from each group and positive control. *$p < 0.05$ vs. control 120 min.

4. Discussion

In the present work we report that aldosterone increases the relaxation mediated by CGRP and RAMP1 expression in SHR mesenteric artery, while CL receptor expression remains unmodified. This effect is dose and time dependent. RU 486 prevents this up-regulation of RAMP1, suggesting that aldosterone affects RAMP1 expression through glucocorticoid receptors activation.

The combination of RAMP1 with CL receptor expression produces a specific and functional CGRP receptor, while expression of the other RAMP family proteins 2 or 3 makes the CL receptor functional for other peptides of the same family such as adrenomodulin or amylin respectively [4]. The mechanism by which RAMP1 makes CL receptor specific for CGRP instead of other substances seems to be glycosylation of some specific CL receptor amino acids. When another RAMP, such as 2 or 3, glycosylates the CL receptor in another position, it loses its specificity for CGRP [13].

Steroids increase the expression of RAMP1 and CL receptor in vascular smooth muscle cells [6]. Sex steroids and pregnancy increase the vasodilatory response to CGRP in mesenteric arteries from rats [9] and that effect is mediated by an increase in the expression of CL receptor and RAMP1 mRNA [10]. Although, we have seen that endogenous female [7] and male [8] sex steroids did not change the functionality of the CGRP system, in a recent work we have reported that aldosterone, through activation of glucocorticoid receptors, decreases the vasoconstrictor response to EFS in endothelium-denuded mesenteric arteries from SHR by increasing the vasodilatory response to CGRP and this effect was time and dose dependent [3]. On the other

hand, the EFS vasoconstrictor response was not modified by aldosterone in WKY, suggesting a specific effect by aldosterone in hypertension [3].

The CGRP vasodilation effect in the mesenteric bed depends on the segment analysed being higher in resistance arteries [14] than in conductance [15] and [16]. The results obtained in the vascular reactivity experiments of the present work indicate that aldosterone did not modify the vasodilator response to CGRP in normotension and led us to conclude that this effect of aldosterone is specific to SHR. With this in mind, it is possible that aldosterone increases the CGRP vasodilation response in SHR mesenteric segments by up-regulating one or both the CGRP receptor components CL receptor and/or RAMP1. The above results indicate that aldosterone 1 µM for 120 min increases the expression of RAMP1 in SHR mesenteric artery, but does not modify CL receptor expression. These modifications were not observed in WKY rats with aldosterone 1 µM 120 min so we performed the rest of the experiments in SHR and concluded that this effect of aldosterone is specific to SHR. The no modification of CL receptor with aldosterone led us to analyse the effect of aldosterone on RAMP1 expression exclusively.

Aldosterone 0.1 µM increased the expression of RAMP1 when the incubation lasted 120 min while aldosterone 0.01 µM did not modify the RAMP1 expression at the same time. Although we observed in a previous work a functional effect of aldosterone on EFS vasomotor response with lower doses and shorter incubation time [3] in the present work the Western blot detects changes in RAMP1 expression at higher doses than functional results. These results indicate that the effect of aldosterone is time and dose dependent and agrees with our earlier functional observations [3].

CL receptor is detected in Western blot in three different forms: a 110 kDa band, a 50 kDa band and a 66 kDa band, representing the mature and functional glycosilated form [17]. We detected the 110 and 66 kDa bands in our experiments but under our experimental conditions the 50 kDa form was not detected. The spectrophotometric analysis revealed that aldosterone did not modify the expression of any of the bands, suggesting that CL receptor does not participate in the increase of the aldosterone mediated vasodilatory response to CGRP in SHR.

RAMP1 is detected in Western blot in two different forms: a 15 kDa band that corresponds to the monomeric form and a 30 kDa band that corresponds to a homodimeric form [18]. It has been suggested that RAMP1, when co-expressed with CL receptor, is only expressed in the 15 kDa form [19]. However, a co-expression of the two forms (30 and 15 kDa) has been described [20] including in vascular tissues [18]. In the present work we detected the 30 kDa form, but under our experimental conditions the 15 kDa form was not detected. The spectrofotometric analysis revealed that preincubation with 120 min of aldosterone 1 µM or 0.1 µM 120 min increased the expression of 30 kDa RAMP1 in mesenteric arteries from SHR.

Since the glucocorticoid receptor antagonist RU 486 prevents the enhanced vasodilatory effect of CGRP elicited by aldosterone, we analysed the possible participation of glucocorticoid receptors in the RAMP1 up-regulation induced by aldosterone. The above results indicate that RU 486 significantly reduces the augmentation of RAMP1 expression induced by aldosterone. RU 486 alone did not modify either the response to CGRP or the basal expression of RAMP1, ruling out any effect of endogenous agonist on CGRP effect or RAMP1 expression.

In summary, aldosterone did not modify the vasodilatory response to CGRP in WKY mesenteric arteries. In SHR, this mineralocorticoid augmented the vasodilation

produced by CGRP by activating glucocorticoid receptors. On the other hand aldosterone did not modify the CL receptor expression, but it did increase the RAMP1 expression only in mesenteric artery from SHR rats by activating glucocorticoid receptors. This up-regulation is time and dose dependent. Since RAMP1 makes CL receptor specific for CGRP, the enhancement of RAMP1 expression seems to be the mechanism by which aldosterone increases the vasodilatory response to CGRP in SHR mesenteric arteries.

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RESUMEN PUBLICACIÓN 4

Objetivos: en este trabajo analizamos el efecto de la aldosterona sobre la relajación al péptido relacionado con el gen d la calcitonina (CGRP) y sobre la expresión de los componentes del receptor de CGRP, "calcitonin like receptor" (CL receptor) y "receptor activity modifying protein 1" (RAMP1) en arterias cerebrales medias de ratas normotensas Wistar Kyoto (WKY) y espontáneamente hipertensas (SHR).

Resultados: el CGRP 0.1nM-0.1μM produjo una relajación dosis dependiente que fue independiente de óxido nítrico (NO) y mayor en SHR que en WKY. La expresión de CL receptor y RAMP1 fue similar en ambas cepas. La relajación a CGRP no fue modificada por la aldosterona en ninguna de las dos cepas, aunque la aldosterona aumento la expresión de CL receptor en SHR, sin que la expresión de RAMP1 fuese alterada.

***Conclusiones*:** el CGRP produce una vasodilatación óxido nítrico independiente mayor en arteria cerebral media de ratas SHR que en WKY, y que es independiente de la expresión de los componentes del receptor de CGRP. Aunque la aldosterona aumenta la expresión de CL receptor en SHR, esto no altera la relajación a CGRP, en tanto que la expresión de RAMP1 permanece sin cambios. Estos resultados indican que, un aumento de la expresión de CL receptor sin un aumento acompañante de RAMP1 no se correlaciona con cambios funcionales en el receptor de CGRP.

Increased expression in calcitonin-like receptor induced by aldosterone in cerebral arteries from spontaneously hypertensive rats does not correlate with functional role of CGRP receptor.

by

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1. INTRODUCTION

Cerebral vascular tone is regulated by a complex set of variables that determine the contractile state of vascular smooth muscle, in which endothelial, myogenic and neural mechanisms play an important role [1]. Cerebral blood vessels are mainly innervated by adrenergic, cholinergic and sensory nerves [1]. The sensory neurotransmitter calcitonin gene-related peptide (CGRP) belongs to the superfamily of calcitonin peptides (calcitonin, CGRPα and β, amylin and adrenomodulin), and plays important roles as a neurotransmitter/neuromodulator in the central nervous system [2]. In the vascular system, CGRP is a potent vasodilator that acts through specific receptors localized in the smooth muscle cells and endothelium [2,3].

The CGRP receptor is a member of the of G-protein coupled receptors (GPCRs) family and is composed of three subunits: a seven transmembrane G protein coupled receptor, called calcitonin-like receptor (CL receptor), the single transmembrane-spanning receptor activity modifying protein 1 (RAMP1) [4] and the receptor component protein (RCP) [5]. The expression of RAMP1 in combination with CL receptor produces a specific and functional CGRP receptor, while the expression of the other RAMP family proteins RAMP2 or RAMP3 respectively makes the receptor specific to other substances like adrenomodulin and amylin [4].

Migraine is a disease that consists in recurrent and severe headache attacks, in which augmented cerebral vessels vasodilation mediated, among others, by CGRP, has been implicated. [1]. On the other hand, several researchers have focused on the interaction between hypertension, aldosterone and migraine attacks [6-9]

In previous studies we have demonstrated that in mesenteric arteries from hypertensive rats, aldosterone increased vasodilator response to CGRP [10] by an up-

regulation of RAMP1 [11]. This effect was not observed in arteries from normotensive rats. Therefore, given the importance of CGRP in the pathogenesis of migraine and its association with aldosterone and hypertension, we hypothesized that aldosterone could modify the vasodilator response to CGRP in cerebral arteries from hypertensive rats.

Taking all into account, our objectives were to analyze whether aldosterone modifies the vasomotor response to CGRP in middle cerebral arteries from normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) and to determine whether this functional effect is associated to alterations in receptor components expression, CL receptor and/or RAMP1.

2. MATERIALS AND METHODS

2.1. Experimental procedure

Male 6-month-old WKY and SHR rats (weighing 200-250 g) were used. After sacrifice by CO_2 inhalation, the brain were carefully removed and placed in Krebs-Henseleit solution (KHS) at 4° C. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85.23, revised in 1996).

2.2. Reactivity experiments

Middle cerebral arteries (MCA) were dissected and segments, 2 mm in length, were mounted in a small-vessel dual-chamber myograph for measurement of isometric tension according to the method described by Mulvany & Halpern [12]. After a 30 min equilibration period, segment contractility was tested by an initial exposure to a high-K^+ solution (120 mM K^+-KHS). Next, concentration-response curves to calcitonin gene-related peptide (CGRP) (0.01 nmol/ L to 0.1 µmol/ L) were performed in arteries previously contracted with a thromboxane A_2 mimetic, U 46 619 (1 µM).

To determine the possible effect of 1 µM aldosterone on CGRP vasodilator response, this mineralocorticoid was added to the media after the first concentration-response curve to CGRP. Four and six hours later, a second and a third CGRP curve was repeated in the presence or absence of aldosterone. The same experiments were carried out in the presence of the nitric oxide synthase inhibitor L-Nitro-aginine-methyl-

ester (L-NAME, 100μM) added 30 minutes before the second CGRP curve to evaluate the participation of endothelium-derived nitric oxide.

At the end of all experiments, segments were thoroughly washed and KHS was replaced by 120 mM K^+-KHS; once the contraction was stable, 0.1 mM papaverine was added. The maximum response of the arteries [1.86 ± 0.17 (n = 10) and 1.54 ± 0.15 mN/mm (n = 8) for WKY rats and SHR, respectively; P>0.05] was determined by the difference between the tone generated by the first exposure to 120 mM K^+-KHS and that produced by 0.1 mM papaverine.

2.3. Western Blot analysis

Middle cerebral arteries from WKY and SHR rats were dissected out and placed on an organ bath with Krebs-Henseleit solution at 37ºC continuously bubbled with 95%O_2 and 5% CO_2 during the experiments. WKY middle cerebral arteries were divided in three groups: a first group was quickly frozen to analyze the basal expression of CGRP receptor components; a second was maintained 6 hours in control conditions; and a third was maintained with aldosterone 1μM for 6 hours. Middle cerebral arteries from SHR were divided into several groups: a first group was quickly frozen to analyze the basal expression of CGRP receptor components; other were maintained 4 or 6 hours in control conditions; and other groups were treated with either aldosterone 1μM for 4 or 6 hours, or aldosterone 0.1μM for 6 hours.

Once incubation had finished, middle cerebral arteries were quickly frozen at -80ºC. For Western Blot analysis of CL receptor and RAMP1 protein expression, middle cerebral arteries were homogenised in a boiling buffer composed of 1mM sodium vanadate (a protease inhibitor), 1% SDS and 0.01M pH 7.4 Tris-HCl. Homogenates containing 16.5 μg protein were electrophoretically separated on a 12%

SDS-polyacrylamide gel (SDS-PAGE) and then transferred to polyvinyl difluoride membranes (Bio Rad Immun-Blot® overnight at 4ºC, 230 mA, using a Bio-Rad Mini Protean III system (Bio-Rad Laboratories, Hercules, CA, USA) containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS. Prestained SDS-PAGE broad range standards (Bio-Rad Laboratories) were used as molecular mass markers. The membrane was blocked for 2 hours at room temperature in Tris-buffered-saline solution 100 mM (0.9% w/v NaCl, 0.1% SDS) with 5% non-fat powdered milk before being incubated overnight at 4ºC with rabbit anti rat RAMP1 (1:250 dilution) or rabbit anti rat CRLR (CL receptor), (1:1000 dilution) antibodies. After washing, the membrane was incubated with a 1:1000 dilution of antirabbit Immunoglobulin G antibody conjugated to horseradish peroxidase. The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminiscence system and subjected to autoradiography (Konica-Minolta films, Japan). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56). The same membrane was used to determine a-actin expression, and the content of the latter was used to correct CL receptor and RAMP1 expression in each sample, by means of an anti a-actin monoclonal antibody (1:3000 dilution). A positive control (rat brain homogenate) for both RAMP1 and CL receptor was used in every experiment.

2.4. Solution and drugs

The composition of KHS was as follows (mM): NaCl 115, $CaCl_2$ 2.5, KCl 4.6, H_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03. The drugs used were: d-aldosterone, CGRP, L-Nitro-arginine-methyl-ester (L-NAME) (Sigma; St.

Louis, MO, U.S.A) and the thromboxane A_2 mimetic, U 46 619 (Calbiochem, Darmstadt, Germany). Drug stock solutions (10 mM) of drugs were made in distilled water, except for aldosterone, which was dissolved in ethanol. These solutions were kept at -20^{o} C and appropriate dilutions were made in KHS on the day of the experiment. The antibodies were CRLR (CL receptor) polyclonal antibody (Alpha Diagnostic International, San Antonio, USA), RAMP1 polyclonal antibody (Santa Cruz Biotechnology inc, Europe), α-actin mouse anti-rat antibody (Sigma; St. Louis, MO, U.S.A) and anti-rabbit horseradish peroxidase antibody (Amersham International plc, Little Chalfont, UK).

3. RESULTS

3.1. Vasodilator response to CGRP

In both strains CGRP (0.1 nM-0.1 μM) produced a concentration dependent relaxation that was significantly higher in SHR (Fig 1). The vasodilator response to a second curve of CGRP remained unmodified when segments from both strains were maintained for 6 hours in control conditions (Fig 2). The incubation with aldosterone 1 μM for 6 hours did not modify the vasodilator response to CGRP (Fig 2). In segments from both strains, the addition of L-NAME 100μM did not modify the relaxation to CGRP in either control conditions or in the presence of aldosterone (data not shown).

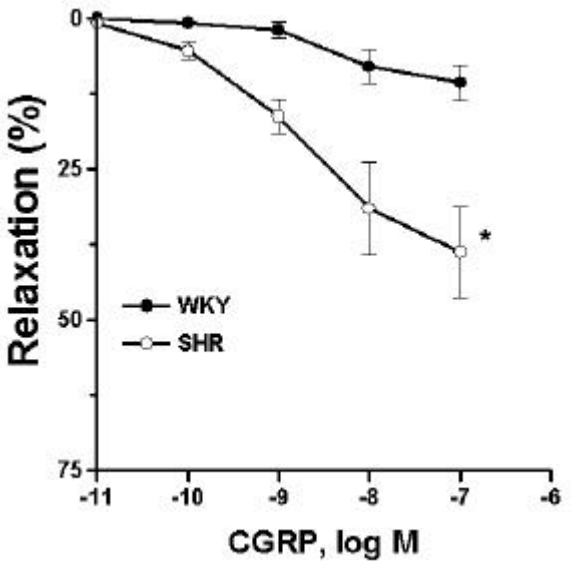

Figure 1. Concentration-dependent relaxation to CGRP in middle cerebral arteries from SHR and WKY rats (n=8-10 each strain) *p<0.05 vs. WKY

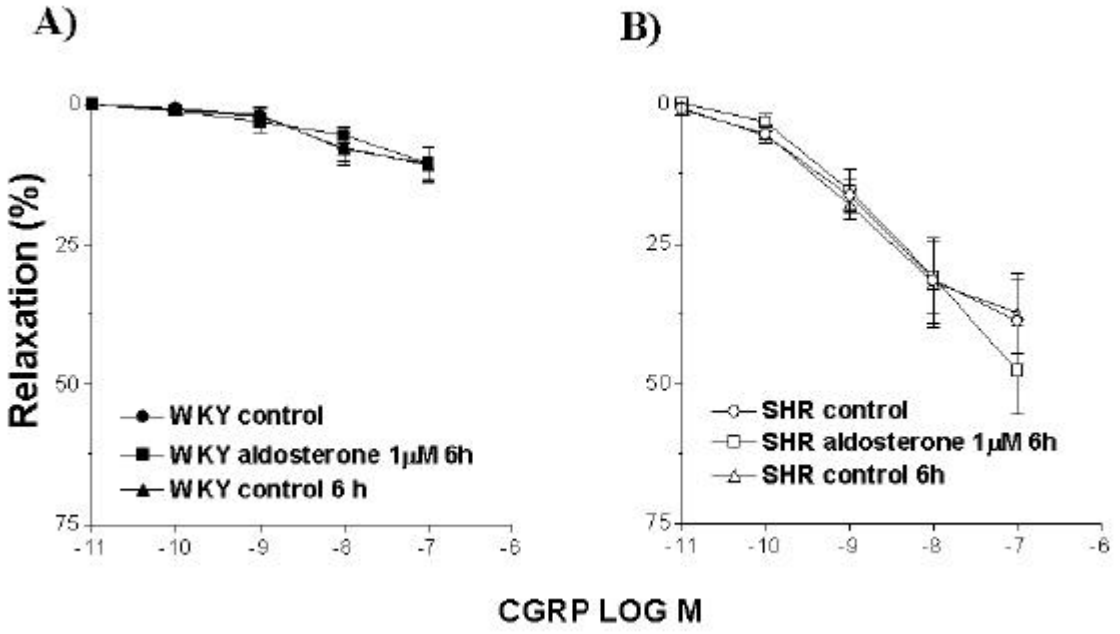

Figure 2. Effects of aldosterone on CGRP mediated relaxation in middle cerebral arteries from WKY (A) and SHR (B) rats. (n=8-10 each strain)

3.2. Western blot analysis

In both strains a 30 KDa band for RAMP1 and a 66 and 110KDa bands for CL receptor were detected in basal conditions. The expression of RAMP1 and CL receptor were not significantly different between SHR and WKY rats (Fig 3).

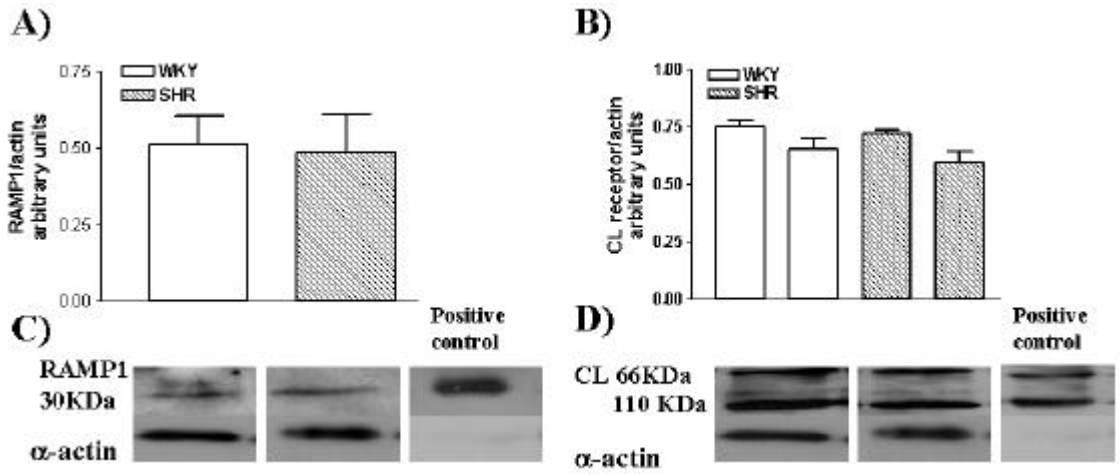

Figure 3. Basal expression of RAMP1 and CL receptor in middle cerebral arteries from WKY and SHR rats. A) and B): Densitometric analysis of RAMP1 30 KDa band and CL receptor 110 and 66 KDa bands respectively. C) and D): Representative immunoblot of cerebral arteries homogenate showing RAMP1 30KDa and CL receptor 110 and 66 KDa bands, α-actin and the positive control. n=4-5 each strain.

In segments from both SHR and WKY rats, incubation with aldosterone 1 μM for 6 hours did not modify the expression of RAMP1 (Fig 4 and 5). Incubation with aldosterone did not modify the expression of CL receptor 66 and 110 KDa bands in arteries from WKY rats (Fig 4), but increased it in arteries from SHR (Fig 5). Aldosterone 1μM during 4 hours or aldosterone 0.1 μM for 6 hours did not modify the expression of RAMP1 or CL receptor (Fig 5).

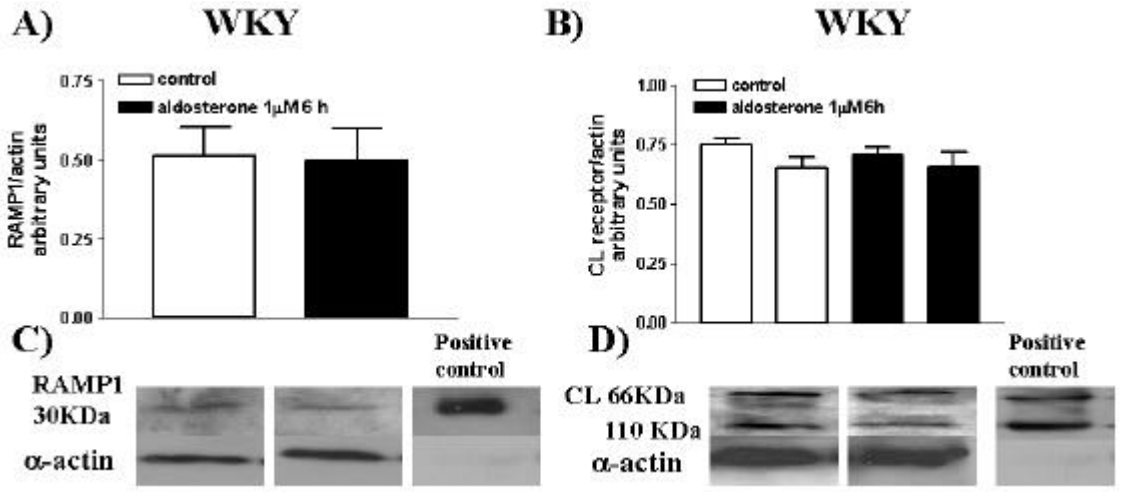

Figure 4 Effects of aldosterone on RAMP1 and CL receptor expression in cerebral arteries from WKY rats. A) and B): Densitometric analysis of RAMP1 30 KDa band and CL receptor 110 and 66 KDa bands respectively. C) and D): Representative immunoblot of cerebral arteries homogenate showing RAMP1 30KDa and CL receptor 110 and 66 KDa bands respectively, α-actin and the positive control. n=4-5 each group.

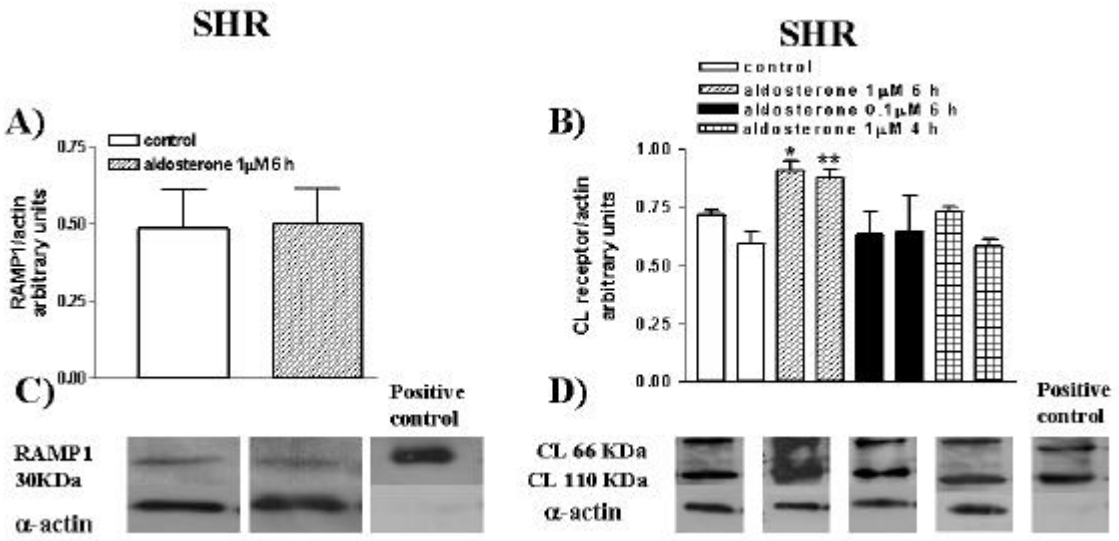

Figure 5. Effects of aldosterone on RAMP1 and CL receptor expression in cerebral arteries from SHR rats. A) and B): Densitometric analysis of RAMP1 30 KDa band and CL receptor 110 and 66 KDa bands respectively. C) and D): Representative immunoblot of cerebral arteries homogenate showing RAMP1 30KDa and CL receptor 110 and 66 KDa bands respectively and the positive control. n=4-5 each group. $^{**}p<0.01$ vs. control; $^{*}p<0.05$ vs. control

4. DISCUSSION

CGRP produced a dose-dependent relaxation in middle cerebral arteries from WKY and SHR, like that observed in other vascular beds, such as mesenteric arteries from WKY and SHR [11]. However, in middle cerebral arteries from SHR, the vasodilator response to CGRP was greater than in WKY rats, in contrast to observed in mesenteric arteries, where the response was similar [12]. In contrast to observed in most vascular beds, where part of the vasodilator effect to CGRP is dependent of endothelium-derived nitric oxide, in middle cerebral arteries from WKY and SHR, vasodilation to CGRP was independent of nitric oxide, as has also been observed in basilar arteries [13].

Cerebral arteries express both CGRP receptor components, CL receptor and RAMP1 [14]. In contrast to observed in other vascular beds, where CGRP response increased in association with increases in CL receptor and RAMP1 expression [15], our results demonstrated that in cerebral arteries from hypertensive rats the enhanced vasodilation to CGRP is probably mediated by mechanisms that are downstream from the CGRP receptor. This mechanism could be an increased production of the second messenger cAMP, as previously reported in cerebral vessels from SHR [16].

The combination of RAMP1 with CL receptor expression produces a specific and functional CGRP receptor, while expression of RAMP family proteins 2 or 3 makes the CL receptor functional for other peptides of the same family, in these cases adrenomodulin or amylin, respectively [4]. The mechanism by which RAMP1 makes CL receptor specific for CGRP, instead of other substances, seems to be the glycosylation of some specific amino acids in the CL receptor. When another RAMP, such as 2 or 3, glycosylates the CL receptor in another position, it loses its specificity for CGRP [17].

It has been described that glucocorticoids [18] and sex steroid hormones [15] increase the expression of both CGRP receptor components, CL receptor and RAMP1, in vascular tissues and that this increase produces an augmented response to CGRP [15]. However, we have reported that an increase in RAMP1 induced by aldosterone, without increasing CL receptor expression, is enough to increase the response to CGRP in mesenteric arteries from SHR [11]

Taking all the above into account, we analysed whether aldosterone modifies the response to CGRP and its receptor expression. Our results indicate that aldosterone increases CL receptor expression in a dose and time dependent manner only in SHR. This result reinforces the fact that the effect of aldosterone on CGRP receptor components expression does not occur in normotension, as observed previously [11]. Surprisingly, this mineralocorticoid did not modify the vasodilator effect of CGRP in middle cerebral arteries from SHR, indicating that the increase of CL receptor expression without an increase on RAMP1 expression cannot enhance the response to CGRP. This result, observed for the first time (in our knowledge) in a complete tissue, confirms the results observed in isolated cells, in which a transfection of CL receptor gene plus a RAMP1 gene leads to the generation of a functional CGRP receptor, while the transfection of CL receptor gene alone leads to a non-functional CGRP receptor [17,19].

In summary, CGRP elicited a greater vasodilation in middle cerebral arteries from SHR than in WKY normotensive rats, and this vasodilation was not accompanied by an increased expression of CGRP receptor components. Aldosterone increased the expression of CL receptor only in SHR, while RAMP1 expression and vasodilation to CGRP were not modified in this strain. These results indicate that the increase in CL

receptor, without an increase in RAMP1, does not correlate with changes in functional role of CGRP receptor.

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CONCLUSIONES

Las conclusiones que podemos extraer de este trabajo son las siguientes:

- El CGRP, que es un potente vasodilatador, participa en la fisipatología de la HTA, por lo que su estudio es interesante para el diseño de terapias antihipertensivas o frente a patologías relacionadas, tales como el vasoespasmo posterior a la hemorragia subaracnoidea o la preeclampsia.
- En arteria mesentérica de rata, la aldosterona produce una disminución de la respuesta vasomotora mediada por EFS, que está mediada por un aumento de la sensibilidad a CGRP. Este fenómeno sólo se produce en SHR, lo que indica que es específico de la HTA. Esto se produce por un aumento de la expresión de proteínas por la activación de receptores glucocorticoideos por parte de la aldosterona. En este aumento de la respuesta vasomotora a CGRP intervienen la activación de canales de potasio ATP dependientes y el cGMP.
- El mecanismo por el cual la aldosterona aumenta la respuesta a CGRP en arterias mesentéricas de SHR parece ser un aumento de la expresión de RAMP1, la cual confiere especificidad al receptor de CGRP para este neuropéptido.
- En arterias cerebrales medias de rata hay una mayor respuesta a CGRP en SHR que en WKY, que no está relacionada con una mayor expresión de los componentes de su receptor. Además, la aldosterona aumenta la expresión de CL receptor sólo en SHR. Sin embargo, este aumento no se ve acompañado de un aumento de la relajación a CGRP, ya que no se aumenta la expresión de RAMP1. De esta manera, se demuestra por primera vez a nivel de tejido completo, que RAMP1 es necesaria para que CL receptor actúe como un receptor funcional para CGRP.

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